Population Structure Analysis of *Phytophthora* species infecting Black Pepper

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By

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This is to certify that all the corrections as suggested by the adjudicators on the thesis submitted by Ms. Fathimath Zumaila titled "Population Structure Analysis of Phytophthora species infecting Black Pepper" has been incorporated. The contents in the hard copy and soft copy are one and the same.

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DECLARATION

I hereby declare that the work presented in the thesis entitled **"Population Structure Analysis of *Phytophthora* species infecting Black Pepper"** is based on the original work done by me under the guidance of Dr. A. Jeevalatha and has not been included in any other thesis submitted previously for the award of any degree. The contents of the thesis have undergone plagiarism check using iThenticate® software at C.H.M.K. Library, University of Calicut, and the similarity index found within the permissible limit. I also declare that the thesis is free from AI-generated contents.

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Fathimath Zumaila

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ABBREVIATIONS AND SYMBOLS

%	Percentage
µM	Micro Molar
ADC	Analogue to Digital Converter
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
ATCC	American Type Culture Collection
BC	Before Christ
bp	Base Pair
CDPK	Calcium Dependent Protein Kinase
cm	centimeter
CRN	Crinkler and Necrosis
CV	Coefficient of Variation
DAMP	Damage-Associated Molecular Pattern
DCL	Dicer-Like
DICE	Diversity Index Calculation and Evaluation
DMRT	Duncan's Multiple Range Test
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
dsRNA	Double Stranded RNA
EDS	Enhanced Disease Susceptibility
EGF	Epidermal Growth Factor
ERIC	Enterobacterial Repetitive Intergenic Consensus
ETI	Effector Triggered Immunity
FACS	Fluorescence-Activated Cell Sorting
FSC	Forward Scatter Channel

GLYK	Glycerate Kinase
h	Hours
H_2O_2	Hydrogen Peroxide
ha	Hectare
ISSR	Inter Simple Sequence Repeat
kb	Kilo Base
l/b	Length/breadth
LAMP	Loop-Mediated Isothermal Amplification
LRR	Leucine-Rich Repeat
MAMP	Microbe-Associated Molecular Pattern
MAPK	Mitogen Activated Protein Kinase
Mbp	Megabase Pair
MEGA	Molecular Evolutionary Genetics Analysis
MGSI	Mycelial Growth Speed Index
Min	Minute
mL	Milliliter
mm	millimeter
mtDNA	Mitochondrial DNA
NBS	Nucleotide Binding Site
ng/µL	Nanogram per Microliter
^oC	degree Celsius
PAMP	Pathogen-Associated Molecular Pattern
PCNB	Pentachloronitrobenzene
PCoA	Principal Coordinate Analysis
PCR	Polymerase Chain Reaction
pg	Picogram
PopART	Population Analysis with Reticulate Trees

PP2A	Protein Phosphatase 2A
PR	Pathogenesis Related
PRR	Pattern Recognition Receptor
PTI	Pattern Triggered Immunity
Pvt Ltd	Private Limited
qPCR	Quantitative Polymerase Chain Reaction
RAMS	Random Amplified Microsatellites
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal DNA
REP	Repetitive Extragenic Palindromic
RFLP	Restricted Fragment Length Polymorphism
RK	Receptor Kinase
RLCK	Receptor-Like Cytoplasmic Kinase
RLP	Receptor-Like Proteins
RMSD	Root Mean Square Deviation
ROS	Reactive Oxygen Species
RPA	Recombinase Polymerase Amplification
RPA-LFD	Loop-Mediated Isothermal Amplification-Lateral Flow Dipstick
RPM	Revolutions Per Minute
RxLR	Arginine-x-Leucine-Arginine
Sec	Second
siRNA	Small Interfering RNA
SNP	Single Nucleotide Polymorphism
spp	Species
SSC	Side Scatter Channel
SSR	Simple Sequence Repeat
STE	Sodium chloride Tris EDTA

t	Tonnes
TAE	Tris Acetate EDTA
U/µL	Units per Microliter
UBC	University of British Columbia
UK	United Kingdom
USA	United States of America
V	Volume

ABSTRACT

Population Structure Analysis of *Phytophthora* species infecting Black Pepper

Black pepper is among the highly valued spice crops worldwide, but several diseases ravage its cultivation. Foot rot is one of the catastrophic diseases caused by a devastating pathogen, *Phytophthora*, which leads to a severe outbreak if left unnoticed. In the present study, the population structure of *P. capsici* and *P. tropicalis* infecting black pepper from black pepper growing areas in Kerala, Karnataka, Tamil Nadu and Goa were studied by characterizing the isolates viz., species identification, distribution and mating type. The isolates obtained were analyzed by PCR assay using Ypt1 gene-based species-specific and ITS primers for species identification. The results showed that *P. capsici* and *P. tropicalis* were the species that were most associated with the disease, though other species were also observed infrequently. The mating type analysis further revealed that all the isolates belong to A1 mating type.

Genome size analysis ensures the robustness of the genetic variation that exists within the population. An extensive comprehension on the genome size would unravel the adaptive ability of the pathogen to extreme environmental conditions, increased pathogenicity and development of fungicide resistance, which would help in streamlining the management approaches. Here, we adopted one of the most reliable techniques, i.e., flow cytometry. It was observed that *P. capsici* had slightly greater genome content than that of *P. tropicalis*. Further, some of the phenotypic characters were analyzed and correlation with the genome size was studied, and it was observed that although there was no significant positive correlation between the variables, a slight negative correlation was observed between the mycelial growth and virulence. However, the pathogens were sensitive to the commonly recommended fungicides to date.

The genetic diversity of *Phytophthora* isolates was resolved using RAMS (Random Amplified Microsatellites) and REP (Repetitive Extragenic Palindromic)-PCR fingerprinting. The population parameters were studied using POPGENE version 1.32 software, which showed the extent of variation that existed within the population. Based on the scoring data of the band produced by RAMS and REP-PCR, UPGMA dendrogram was plotted, which broadly divided the isolates into four clusters. *P. capsici* and *P. tropicalis* were grouped in separate clusters, i.e., in sub-

clusters I, II and III, and IV, respectively. This was also confirmed by PCoA analysis. The isolates displayed varied morphology irrespective of the clusters generated, and further, the cross-infectivity analysis showed that the black pepper isolates were capable of infecting crops like tomato, cucumber, pumpkin, nutmeg and chilli and a few isolates infected cardamom *in vitro*. No symptoms were observed in vanilla, coconut and arecanut.

Haplotype analysis was carried out using mitochondrial (Cox1, Cox2, Nad1 and Nad5) and nuclear genes (β-tubulin, EF-1α, Enolase, HSP90, TigA and Ura3). The morphological characterization of isolates based on species was found to be unreliable due to the presence of coinciding characters between the species. Various genetic diversity parameters and demographic studies were performed using DnaSP v6.12.03. Among the isolates studied, the greater number of haplotypes was observed for EF1-α and that of Nad1 and Ura3 were less. The haplotype network analysis performed with the black pepper isolates and *P. capsici* infecting diverse hosts from Hawaii and some of the contiguous United States showed that the Indian population is rooted in the US population. Further, the population bottleneck event and its restoration were revealed by the demographic analysis.

This study also attempted to study the expression of various RxLR effectors by real-time PCR and two of the highly expressed RxLR genes during the infection, i.e., RxLR29 and RxLR132, were selected for further studies. The three-dimensional structures of two of the RxLR proteins were resolved using homology modelling. Moreover, its interacting counterpart was selected based on earlier reports and protein-protein docking studies were performed. The results showed the existence of biologically feasible interactions between the proteins, i.e., RxLR29 and DRB4 and RxLR132 and CMPG1.

Keywords: Black pepper*, Phytophthora capsici, P. tropicalis*, population, genome size, haplotype, effectors.

സംഗ്രഹം

കുരുമുളകിനെ ബാധിക്കുന്ന *ഫൈറ്റോഫ്തോര* സ്പീഷീസുകളുടെ ജനസംഖ്യ ഘടനാ വിശകലനം

കുരുമുളക് ലോകമെമ്പാടും ഉയർന്ന മൂല്യമുള്ള സുഗന്ധസസ്യങ്ങളിൽ ഒന്നാണ്, എന്നാൽ പല വ്യാധികളും അതിന്റെ കൃഷിയെ തകർക്കുന്നു. ഫൂട്ട്രോട്ട് എന്നത് ഗുരുതരമായ ഒരു രോഗമാണ്, അത് *ഫൈറ്റോഫ്തോര* എന്ന നാശകമായ രോഗാണു മൂലം സംഭവിക്കുന്നു, ശ്രദ്ധിക്കാതെ പോയാൽ വൻതോതിലുള്ള ബാധയ്ക്കു കാരണമാകുന്നു. കുരുമുളക് വളർത്തുന്ന കേരളം, കർണാടക, തമിഴ്നാട്, ഗോവ എന്നിവിടങ്ങളിൽ നിന്ന് ശേഖരിച്ച *ഫൈറ്റോഫ്തോര കാപ്സിസിയും ഫൈറ്റോഫ്തോര ട്രോപിക്കാലിസ്ഉം* ബാധിച്ച കുരുമുളക് സാന്ദ്രതകളുടെ ജനസംഖ്യ ഘടന ഈ പഠനത്തിൽ വിശകലനം ചെയ്തു. ശേഖരിച്ച ഐസൊലേറ്റുകൾ സ്പീഷീസ് തിരിച്ചറിയൽ, വ്യാപനം, മേറ്റിങ് തരം എന്നിവയുടെ അടിസ്ഥാനത്തിൽ വിശകലനം നടത്തി. സ്പീഷീസ് തിരിച്ചറിയലിന് Ypt1 ജീൻ അടിസ്ഥാനമാക്കിയ സ്പീഷിസ്-സ്പെസിഫിക് പ്രൈമറുകളും ITS പ്രൈമറുകളും ഉപയോഗിച്ച് പി.സി.ആർ പരീക്ഷണം നടത്തി. ഫലങ്ങൾ കാട്ടുന്നത് *ഫൈറ്റോഫ്തോര കാപ്സിസിയും ഫൈറ്റോഫ്തോര ട്രോപിക്കാലിസ്ഉം* ആണ് രോഗവുമായി കൂടുതൽ ബന്ധപ്പെട്ടു കാണപ്പെട്ടത്, എങ്കിലും മറ്റു സ്പീഷിസുകളും അപൂർവമായി കണ്ടു. മേറ്റിങ് ടൈപ്പ് വിശകലനം നടത്തുമ്പോൾ എല്ലാ ഐസൊലേറ്റുകളും A1 മേറ്റിങ് ടൈപ്പിലാണെന്ന് കണ്ടെത്തി.

ജനിതക വ്യത്യാസത്തിന്റെ സ്ഥിരത ഉറപ്പാക്കുന്നതിന് ജീനോം വലുപ്പ വിശകലനം പ്രധാനമാണ്. ജനസംഖ്യയിലുള്ള വൈവിധ്യത്തെക്കുറിച്ചുള്ള ഗഹനമായ അറിവ്, പരിസ്ഥിതി മാനദണ്ഡങ്ങളോടുള്ള പകർച്ചക്കാരന്റെ അളവില്ലാത്ത അനുസരണശക്തിയും, രോഗകാരിതയിലുണ്ടാകുന്ന വർധനവും, ഫംഗിസൈഡ് പ്രതിരോധത്തിന്റെ രൂപീകരണവും ഉൾപ്പെടെ പല മേഖലകളിലും ഉൾവെച്ച് നിയന്ത്രണ സമീപനങ്ങൾ ലളിതമാക്കാൻ സഹായിക്കും. ഈ പഠനത്തിൽ, ഏറ്റവും വിശ്വാസയോഗ്യമായ സാങ്കേതിക വിദ്യയായ ഫ്ലോ സൈറ്റോമെട്രി ഉപയോഗിച്ചു.

വിശകലനഫലങ്ങളിൽ *ഫൈറ്റോഫ്തോര കാപ്സിസിയ്ക്ക്* *ഫൈറ്റോഫ്തോര ട്രോപിക്കാലിസ്നെ* അപേക്ഷിച്ച് ചെറുതായി കൂടിയ ജീനോം ഉള്ളടക്കം കാണപ്പെട്ടു. കൂടാതെ, ചില ഫിനോട്ടൈപ്പിക സ്വഭാവങ്ങൾ പരിശോധിക്കുകയും അവ ജീനോം വലുപ്പവുമായി ബന്ധിപ്പിച്ച് പഠിക്കുകയും ചെയ്തു. മാറ്റങ്ങൾക്കിടയിൽ ഗണ്യമായ പോസിറ്റീവ് സഹപരിസരം കണ്ടുവരാത്തതിനുശേഷവും, മൈസീലിയൽ വളർച്ചയും രോഗകാരിതയും തമ്മിൽ ചെറുതായി നെഗറ്റീവ് സഹപരിസരം കാണപ്പെട്ടു. എങ്കിലും, ഈ പകർച്ചകാരികൾ ഇന്നുവരെയുള്ള സാധാരണ ഫംഗിസൈഡുകൾക്ക് അനുയോജ്യമായ പ്രതികരണം കാഴ്ചവച്ചു.

ഫൈറ്റോഫ്ഥോറ ഐസൊലേറ്റുകളുടെ ജനിതക വൈവിധ്യം RAMS (റാൻഡം ആംപ്ലിഫൈഡ് മൈക്രോസാറ്റലൈറ്റ്) REP-PCR (റിപ്പറ്റേറ്റീവ് എക്സ്ട്രാജീനിക് പാലിൻഡ്രോമിക് PCR) ഫിംഗർപ്രിന്റിംഗ് വഴി മനസിലാക്കി. ജനസംഘ്യാ ഘടനാ പാരാമീറ്ററുകൾ POPGENE പതിപ്പ് 1.32 സോഫ്റ്റ്വെയർ ഉപയോഗിച്ച് പഠിച്ചപ്പോൾ, ജനസംഖ്യയ്ക്കുള്ളിൽ നിലവിലുണ്ടായിരുന്ന വ്യത്യാസങ്ങളുടെ വ്യാപ്തി വ്യക്തമാകുന്നു. RAMS-നും REP-PCR-നും വഴി ഉണ്ടാക്കിയ ബാൻഡുകളുടെ സ്കോറിംഗ് ഡാറ്റയുടെ അടിസ്ഥാനത്തിൽ UPGMA ഡെൻഡ്രോഗ്രാം നിർമ്മിക്കപ്പെട്ടു. ഇതിൽ, ഐസൊലേറ്റുകൾ വലിയ തോതിൽ നാല് ക്ലസ്റ്ററുകളിലായി വിഭജിക്കപ്പെട്ടു. *ഫൈറ്റോഫ്തോര കാപ്സിസിയും* *ഫൈറ്റോഫ്തോര ട്രോപിക്കാലിസ്ഉം* പ്രത്യേകം ക്ലസ്റ്ററുകളായി, അതായത് ഉപക്ലസ്റ്ററുകൾ I, II, III *ഫൈറ്റോഫ്തോര കാപ്സിസിയും*, IV *ഫൈറ്റോഫ്തോര ട്രോപിക്കാലിസ്ഉം* എന്നിങ്ങനെയാണ് ചേരുന്നത്. ഈ ഫലങ്ങൾ PCoA വിശകലനം കൊണ്ടും സ്ഥിരീകരിക്കപ്പെട്ടു. ക്ലസ്റ്ററുകളുടെ അടിസ്ഥാനത്തിൽ വേറിട്ട സ്വഭാവം കാണിച്ചെങ്കിലും, ഐസൊലേറ്റുകൾ വ്യത്യസ്ത മോർഫോളജി പ്രകടിപ്പിച്ചു. ക്രോസ് ഇൻഫെക്ടിവിറ്റി വിശകലനം പ്രകാരം കുരുമുളക് ഐസൊലേറ്റുകൾ തക്കാളി, വെള്ളരിക്ക, മത്തങ്ങ, ജാതിക്ക, മുളക് മുതലായ വിളികളെ ആക്രമിക്കാൻ കഴിയുന്നവയാണെന്ന് കാണപ്പെട്ടു. ഇൻ വിത്രോ സാഹചര്യത്തിൽ ഏറെ കുറച്ച് ഐസൊലേറ്റുകൾ ഏലം പണിപ്പാടിനെയും ബാധിച്ചു. എന്നാൽ വാനില, തേങ്ങ, അടക്ക എന്നീ സസ്യങ്ങളിൽ രോഗ ലക്ഷണങ്ങൾ കണ്ടില്ല.

ഹാപ്ലോട്ടൈപ്പ് വിശകലനം മൈറ്റോകോണ്ട്രിയൽ ജീനുകൾ ആയ Cox1, Cox2, Nad1, Nad5 കൂടാതെ ന്യൂക്ലിയർ ജീനുകൾ ആയ β-tubulin, EF-1α, Enolase, HSP90, TigA, Ura3 എന്നിവ ഉപയോഗിച്ച് നടന്നു. പകർച്ചകാരികളുടെ സ്പീഷീസ് അടിസ്ഥാനമാക്കിയുള്ള രൂപപരമായ വിശകലനം വിശ്വാസയോഗ്യമല്ലെന്നത് കണ്ടെത്താനായി, കാരണം പല സ്പീഷീസിനും തമ്മിൽ ഒത്തു പോകുന്ന ലക്ഷണങ്ങൾ കാണപ്പെട്ടു. ജനിതക വൈവിധ്യ സൂചകങ്ങളും ജനസംഖ്യാ പഠനങ്ങളും DnaSP പതിപ്പ് 6.12.03 ഉപയോഗിച്ച് നടന്നു. പഠനത്തിലെടുത്ത ഐസൊലേറ്റുകളിൽ, ഏറ്റവും കൂടുതലായ ഹാപ്ലോട്ടൈപ്പുകൾ EF-1α ജീനിനായിരുന്നു, അതേസമയം Nad1യും Ura3യും ഏറ്റവും കുറവായിരുന്നു. കുരുമുളക് ഐസൊലേറ്റുകളും, ഹവായിയും അമേരിക്കയിലെ മറ്റ് ഭാഗങ്ങളിൽ നിന്നും വിവിധ ഹോസ്റ്റുകളെ ബാധിക്കുന്ന *ഫൈറ്റോഫ്തോര കാപ്സിസി* ഐസൊലേറ്റുകളും ഉൾപ്പെടുത്തി നടത്തിയ ഹാപ്ലോട്ടൈപ്പ് നെറ്റ്വർക്ക് വിശകലനം പ്രകാരം, ഇന്ത്യയിലെ ജനസംഖ്യ അമേരിക്കൻ ജനസംഖ്യയിൽ നിന്ന് ഉത്ഭവിച്ചതായി കാണപ്പെട്ടു. അതോടൊപ്പം, ജനസംഖ്യാ ദൗർബല്യഘട്ടവും (bottleneck) അതിന്റെ പുനസ്ഥാപനവും ജനസംഖ്യാ വിശകലനം വെളിപ്പെടുത്തുന്നു.

ഈ പഠനത്തിൽ വിവിധ RxLR എഫക്ടർമാരുടെ രേഖപ്രഖ്യാപനം റിയൽ ടൈം PCR ഉപയോഗിച്ച് പരിശോധിക്കാൻ ശ്രമിച്ചു. അണുബാധ സമയത്ത് ഉയർന്ന രേഖപ്രഖ്യാപനം കണ്ട രണ്ടുവിവിധ RxLR ജീന്മാർ ആയ RxLR29, RxLR132 എന്നിവ കൂടി തുടർ പഠനങ്ങൾക്ക് തെരഞ്ഞെടുത്തു. ഈ രണ്ടു RxLR പ്രോട്ടീനുകളുടെയും ത്രിമാന ഘടനകൾ ഹോമോളജി മോഡലിംഗ് വഴി നിർവ്വചിച്ചു. അവയുടെ ഇടനില സഹപ്രവർത്തകനായി മുൻ റിപ്പോർട്ടുകൾ അടിസ്ഥാനമാക്കി DRB4 (RxLR29-നൊപ്പം) മേൽക്കാരി, CMPG1 (RxLR132-നൊപ്പം) എന്നിവ തിരഞ്ഞെടുക്കുകയും പ്രോട്ടീൻ-പ്രോടീൻ ഡോക്കിംഗ് പഠനങ്ങൾ നടത്തുകയും ചെയ്തു. ഫലങ്ങൾ പ്രകാശിപ്പിക്കുന്നത്, RxLR29-DRB4, RxLR132-CMPG1 എന്നിങ്ങനെ ജീവശാസ്ത്രീയമായി സാധുവായ ഇടപെടലുകൾ നിലവിലുണ്ടെന്നും ആണ്.

സൂചക ശബ്ദങ്ങൾ: കുരുമുളക്, *ഫൈറ്റോഫ്ഥോറ കാപ്സിസി, ഫൈറ്റോഫ്ഥോറ ട്രോപിക്കാലിസ്*, ജനസംഖ്യ, ജീനോം വലുപ്പം, ഹാപ്ലോടൈപ്പ്, എഫക്ടറുകൾ.

CHAPTER 1
INTRODUCTION

Black pepper (*Piper nigrum* L.) is one among the most valuable spice crops worldwide due to its key role in medicine and condiment. India is the fifth largest producer of black pepper (World Population Review, 2024) and Karnataka, the leading producer, cultivates about 222,394 ha of land and produces 89,000 t, Kerala, 27,505 t in 73,615 ha and Tamil Nadu cultivates 7,651 ha with 2,562 t of produce (Spices Board, 2025). Other states include Maharashtra, North eastern states and Andaman and Nicobar Islands (Devasahayam *et al.,* 2015). The decline in black pepper production can be attributed to reduced area of production, poor cultivation practices and diseases (Rajshree *et al.,* 2023). Nursery diseases of black pepper include foot rot, leaf rot and blights and in plantations, anthracnose, phyllody and stunted disease occur (Anandaraj & Sarma, 1995). According to a recent survey by the Agriculture Department, the production loss of black pepper in Kerala caused due to rain and flood in 2018-2019 accounts for about 10,700 tons (ICAR-Indian Institute of Spices Research, 2023) and the presence of *Phytophthora*, the causal agent of foot rot disease was identified from the soil sample.

The identification of foot rot disease in India dates back to 1902 and was reported by Barber and Butler (Sarma & Anandaraj, 1997). *Phytophthora* is an oomycete soilborne plant pathogen. It has aseptate coenocytic mycelium and it bears motile zoospores within the sporangium (Erwin & Ribeiro, 1996). According to the key of Waterhouse (1963*), P. capsici* and *P. tropicalis* fall in group II, which was characterized by a prominent papilla at the apex of the sporangium. Members of group II are heterothallic and produce amphigynous antheridia. Other features include caducous sporangia and pedicel length exceeding 20 µm. *P. tropicalis* produces chlamydospores, whereas *P. capsici* does not. The temperature favourable for its growth ranges from 25^{o}C to 30^{o}C (Mehrotra & Aggarwal, 2001). The notable features of *Phytophthora* which prevent them from grouping as true fungi, include tubular cristae of mitochondria, β-glucan cell wall, carbohydrate storage in the form

of mycolaminarin, they do not synthesize sterols and are resistant to polyene antibiotics (Erwin & Ribeiro, 1996).

P. capsici infection was reported in various crops such as eggplant fruit, cacao, cotton balls, black pepper, tomato (Erwin & Ribeiro, 1996), pumpkin, squash (Isakeit, 2007), cucumber and melon (Herrero *et al.,* 2002). *P. tropicalis* was reported in ornamentals like *Epipremnum aureum* and *Hedera helix* (Orlikowski *et al.,* 2006), golden pothos (Leahy, 2006) *Pieris japonica,* a flowering plant (Redekar *et al.,* 2020), *Rhododendron catawbiense* (Hong *et al.,* 2006), breadfruit (Cerqueira *et al.,* 2006), cacao (Chávez-Ramírez *et al.,* 2021; Madhu *et al.,* 2023) and black pepper (Bhai *et al.,* 2022). Apart from *P. capsici* and *P. tropicalis*, *P. nicotianae* was also reported to have an association with foot rot disease of black pepper (Drenth & Guest, 2004; Thao *et al.,* 2024).

The foot rot is one of the serious diseases of black pepper, as it is not confined to a particular part of the plant, and the tender plant part is more vulnerable. The disease is mainly observed in the monsoon season. Once the disease goes unnoticed, the entire vine vanishes in a short period. The disease is characterized by a dark brown water-soaked lesion with a fimbriate margin on leaves and spreads quickly to the nearby tissues, causing necrosis, but collar rot and root rot are extremely lethal to the vine. Since the pathogen is soilborne, the pathogen will survive in the soil from the infected dead vines. The survival structures and infected debris get washed away by water and act as a source of inoculum to infect a healthy plant (Sarma & Anandaraj, 1997). Foliar infection is observed during August and continues up to November. For a successful infection to occur, there must be sufficient availability of rainfall ranging from 15.8 to 32 mm, relative humidity of 91 to 99 % and temperature in the range of 22.7 to 29.6^{o}C and sunlight of 2.8 to 3.5 hours (Mehrotra & Aggarwal, 2001).

P. capsici and *P. tropicalis* are heterothallic in nature. When a single culture is inoculated on media, they do not produce oospores unless there is a presence of another culture which is of the opposite mating type. Oospores are thick-walled structures predominantly produced by the fusion of gametangia, namely antheridia

and oogonia. The thick wall of oospores serves as a resting structure which can withstand adverse environmental stress and remain viable and dormant until they encounter a favourable condition to germinate and flourish.

Study of the genome size of a pathogen allows researchers to understand its genetic complexity and to identify key genes related to pathogenicity. Larger genomes often indicate a greater capacity for genetic diversity and potential virulence factors. The genome size, which is the quantity of DNA of an organism present in a haploid nucleus, is commonly expressed in picograms. According to the neutral theories, genome size is mainly influenced by genetic drift, and natural selection has a minimal role (Blommaert, 2020). The mutations that occur due to transposable elements are one of the major reasons for the increase in genome size (Muszewska *et al.,* 2019). In *Phytophthora*, the genes related to pathogenicity were located in the gene-sparse transposable element-rich regions (Mandal *et al.,* 2022), and this confers adaptability to the pathogen (Engelbrecht *et al.,* 2021). The genetic variations mediated by transposable elements play a major role in the fungicide resistance of the pathogen, and preserving the alleles and their combinations which favour the fungicide resistance urges the need to introduce novel fungicides. Mitochondrial DNA also carries mutations related to the virulence and fungicide resistance of the pathogen (Zaccaron & Stergiopoulos, 2024).

The genetic diversity studies and haplotype analysis help in the better understanding of the pathogen population. It reveals the genotypes (whether it is unique or mixed) that exist within a population. The phylogenetic and genetic genealogy studies trace back its ancestry and give us information on the population migration as well. Precise knowledge of the genetic diversity of the pathogen is necessary in the development of tolerant varieties and to protect it on a long-run basis (Pandey *et al.,* 2023). An in-depth knowledge on the pathogen will impact the host plant in shaping its genetic structure (Kumar & Verma, 2019).

Phytophthora encodes for several avirulence genes which play an active role in the infection process of the host as it activates the effector-triggered immunity (ETI). Due to the selection pressure, there exists a significant diversity in the avirulence

genes which results in the amino acid changes or truncated protein, which makes it obscure for the R protein interaction (Wang *et al.,* 2011). Diversity studies in effector genes are essential as they help in resistance plant breeding (Morales *et al.,* 2020) and to have a better understanding on the host-pathogen coevolution.

Though a lot of diseases have been identified in black pepper, the foot rot disease demands utmost care in managing the disease. Frequent monitoring of the vines was recommended to keep the disease in check. Once the disease has been spotted, oftentimes the characteristics of the lesion give an idea about the pathogen associated with it. To design an effective management strategy, it is necessary to have a broad knowledge of the pathogen, its life cycle, the mechanism of disease development in plants, the fungicide sensitivity, the optimum growth conditions of the pathogen, its behaviour in the environment i.e., the factors that favour the disease, mode of transmission, pattern of virulence. It is also essential to know the genetic diversity of the population, which gives insight into the mutational changes which, in most cases, favour the increased virulence and better adaptability of the pathogen. Hence, the objectives of the present study were formulated in this respect, which ought to provide a better knowledge on the pathogen population.

Objectives

1. To analyze species distribution and mating types of *Phytophthora* species infecting black pepper.

2. To determine the genome size of *Phytophthora* isolates and to study its correlation with virulence and fungicide resistance.

3. To analyze genetic diversity in *Phytophthora* isolates infecting black pepper.

CHAPTER 2
REVIEW OF LITERATURE

2.1. Black pepper

Piper nigrum L., commonly known as black pepper, was also referred to as "King of Spices" and "Black Gold". This was originated at the Malabar coast of Kerala, India. According to archaeological evidence, the consumption of black pepper dates back to 2000 BC. It has been one of the highly valued trade commodities since Medieval times. It is mainly used as a preservative and condiment and also used in medicine (International Pepper Community, 2024).

Fig. 2.1. Black pepper vine

Black pepper, a perennial woody climber (Fig. 2.1), is one of the most valued spice crops worldwide. Black pepper cultivation favours humid and tropical climates. The plant prefers shade over light as it has poor tolerance towards heat. It requires an annual rainfall of about 2000-4000 mm, a temperature range of 23-33^{o}C and 60-95

% humidity for its optimum growth (Kumar *et al.,* 2021). Black pepper vines were often trailed on a support column or on other crops like cardamom, coffee or tea (Kumar *et al.,* 2021; Ravindran & Kallupurackal, 2012). It has alternate phyllotaxy. Black pepper fruit is a drupe, which are often called as berries with a size of about 5 mm in diameter (Berry, 2023; Ravindran & Kallupurackal, 2012).

According to World Population Review (2022), the world leading producer of pepper (*Piper* spp.) is Vietnam which accounts for about 272234.66 t and export quantity of 195978.42 t, followed by Brazil which produces 128331 t and exports 86385.49 t and Indonesia with 81962 t produce and forth largest exporter after United Arab Emirates, where the former accounts for 29580.46 t and the latter exports 30361.68 t. India ranks fifth among the global black pepper producers and exporters, producing 64204.7 t and exporting 19968.48 t. Import statistics show that the United States of America is the largest importer of about 87619.36 t. India and Vietnam stand in second and third position with an import estimate that accounts for about 43475.42 t and 33201.24 t, respectively.

In India, pepper was cultivated majorly in three states in the southern region, namely, Karnataka, Kerala and Tamil Nadu. However, it is also grown in Goa and Maharashtra. Karnataka was the leading producer of pepper consistently from 2018 to 2023 with a total production of 50000 t which were cultivated over 211975 hectares of land and Kerala is the second largest producer with an estimate of 27654 t cultivated over 73732 hectares of land followed by Tamil Nadu which produced about 1642 t over 7465 hectares (Spices Board, 2024). The recent data (2022-2023) shows that the export quantity of pepper from India is 17,958 t, which was decreased from that of 2020-2021 reports (Spices Board, 2024). The United States of America is the greatest importer of Indian pepper, followed by the United Kingdom, Sweden and Germany (Spices Board, 2024).

The black pepper cultivation constraint is broadly divided into four factors. The first one is cultivation constraints, which includes increased cultivation cost, low productivity, replanting problems, drought, poor genotypic quality, adverse climates and pests and pathogen attacks. The second one is the labor constraints, which

includes wage expenses, lack of laborers and/ work cultures, outdated laws and indiscipline. The third factor is trade constraints, which includes high competition, low selling price, poor marketing and seasonal productions. The fourth factor is economic constraints, which include fluctuations in the pricing, declined economic activity, indebtedness and decreased demand in local markets (Sivadas, 2019).

Regardless of the fact that black pepper originated from Kerala, Karnataka leads in terms of area of cultivation and production. When we compare the Spices Board data of 2021-22 and 2022-23, the black pepper produce has been reduced drastically from 55,600 t to 50,000 t in Karnataka, from 32,500 t to 27,654 t in Kerala and from 1,500 t to 1,642 t in Tamil Nadu. One of the several factors that contribute for the decline in production is the pests and pathogens that attack black pepper. The insects that infect black pepper include pollu beetle, pepper stem borer, pepper whitefly, root mealy bugs, leaf gall thrips, top shoot borer and scale insects. Other diseases include wilt, foot rot, nematode, anthracnose and stunt disease (Talucder *et al.,* 2020). One of the most important production constraints among several others in the black pepper cultivation is the foot rot disease of black pepper, which is discussed below.

2.1.1. Foot rot disease

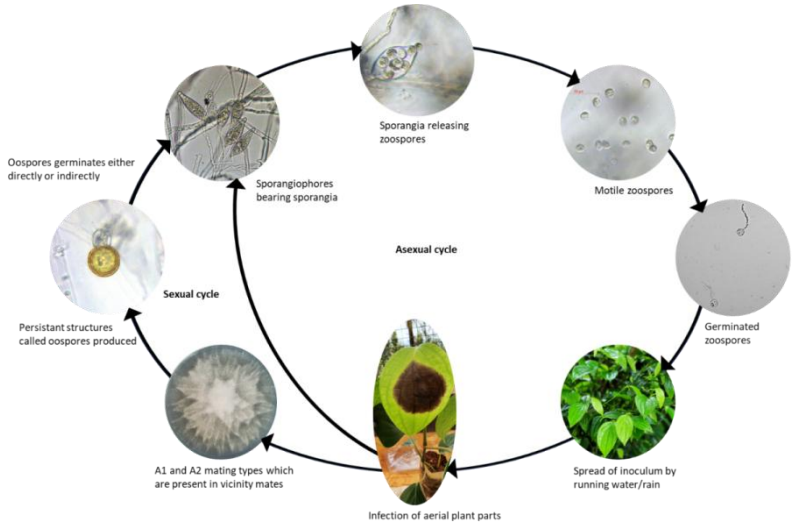


Fig. 2.2. Life cycle of *P. capsici.* (each stage was photographed *in vitro* for illustration purpose).

The causative agents associated with the foot rot disease are *P. capsici* and *P. tropicalis* (Bhai *et al.,* 2022), which are soil-borne in nature. The disease is wet-weather favoured, so its occurrences are mainly observed during the monsoon. *Phytophthora* sporulates under favourable conditions and the motile zoospore acquires the rainwater as the medium for spreading. The crown region is more prone to disease as it is close to the soil surface and it advances towards the tip of the plant. In the soil phase, the disease goes unnoticed until it renders sufficient damage to kill the plant (Sarma & Anandaraj, 1997). The life cycle of *P. capsici* is depicted in Fig. 2.2. Both sexual and asexual modes of reproduction were observed in the pathogen. The mating types viz., A1 and A2 participate in the process to produce oospores, which have the capacity to withstand extreme environmental conditions and can survive more than 3 years, thereby act as the source of primary inoculum in the field (Babadoost & Pavon, 2013). The oospores then germinate either directly by producing the germ tube or indirectly by sporangia formation. Sporangia are also produced as a result of asexual reproduction; the sporangia produced by *P. capsici* and *P. tropicalis* were caducous, which aids the disease spread. These were the papillate structures that harbors 20-40 motile biflagellated zoospores (Barchenger *et al.,* 2018; Quesada-Ocampo *et al.,* 2023) and were released during favourable conditions. The zoospores were then carried away by the rain droplets to reach the host tissue and initiate the infection (Ristaino & Johnston, 1999).

Fig. 2.3. The characteristic lesions inflicted by a) *P. capsici*; b) *P. tropicalis.*

P. tropicalis which was considered as a sub group of *P. capsici* back then was later described as separate species and there exists a notable difference in the characteristics of the lesion inflicted by both the species on the black pepper leaves (Fig. 2.3). The lesion produced by *P. capsici* is dark brown water-soaked lesion with a progressive fimbriate margin and those infected by *P. tropicalis* is dark brown lesion restricted by a yellow halo (Bhai *et al.,* 2022).

2.1.2. Isolation of *Phytophthora*

Phytophthora capsici is a hemibiotrophic organism that exhibits a biotrophic phase of infection initially, where they extract the nutrients from the fresh and lively tissues and later on, they switch to a necrotrophic phase, which leads to the death of the tissue (Barraza *et al.,* 2022). These act as primary invaders of the plant and their isolation is mainly preferred from the freshly infected tissue rather than decayed as the quantity of live mycelia was hard to recover from the dead tissues (Drenth & Sendall, 2001).

From the infected tissues, *Phytophthora capsici* can be isolated by a method known as tissue planting. Here, the tissue is washed to remove surface debris and placed in sterile water where the tissue is being cut in such a manner that it has a part of infected and healthy tissue. These were then blotted and placed onto amended media composed of pentachloronitrobenzene (PCNB), rifampicin, ampicillin, pimaricin and hymexazol. The growing mycelial tips were then transferred to fresh carrot agar media (Akter *et al.,* 2007). Alternative methods include placing the surface-sterilized tissue under light for sporulation, the sporulated tissue was surface sterilized and placed on selective media (Drenth & Sendall, 2001).

Another baiting method for *Phytophthora* infecting *Hevea brasiliensis* proposed by Chee and Foong (1968) includes inoculating a fresh cocoa pod with the infected tissues to the exposed healthy tissue by making a cut. Infection initiates by 96 to 120 h after incubation. The infected tissue was placed onto the selective media for the mycelial growth.

P. capsici from the infected soil samples were isolated by baiting. The baiting on fruit method includes placing the fruit (tomato, brinjal or chilli) on a Petri plate containing the infected soil sample. Fungal growth that appeared after the incubation at room temperature for two to three days was observed under the microscope and was cultured in the aforementioned amended media (Akter *et al.,* 2007).

Yet another method also being widely used to isolate *Phytophthora capsici* from infected soil samples is by baiting with leaves. Here, the soil samples were taken in tubes and were covered with sterile pieces of cloth. Sterile water was then added to the soil such that the pieces of cloth stayed 1 cm below the level of water. Pieces of healthy leaves were then placed on the surface of the water and incubated for 24 h. The leaf bits were then placed on to the amended media after blotting. The spotted mycelia were transferred to fresh carrot agar media (Akter *et al.,* 2007).

Few more alternative techniques used for baiting *Phytophthora* from soil includes planting rooted cuttings or seeds in soil and infection is stimulated by excessive watering, placing the infected tissue or soil sample to the bore cut on a healthy fruits like apple, pear, cocoa or watermelon and a simple and popular technique baiting by immersing partially (leaf bits or fruit pieces) in to the suspension of soil and water (Drenth & Sendall, 2001).

2.1.3. Maintenance of *Phytophthora* cultures

Maintenance of *Phytophthora* cultures are crucial for research purposes. Continuous subculturing can preserve the pathogen, but the virulence features get attenuated; also, it is a tedious job. There are certain methods adopted for the prolonged storage of *Phytophthora* cultures by preserving its vigor. Storing the mycelial discs in vials containing sterile water is a promising method and reports say that viable cultures were revived even after seven years. However, passing into the host is recommended to maintain pathogen vigor. In the case of homothallic species, soybean, hemp or corn seeds can be added to the vial for oospore induction (Drenth & Sendall, 2001; Sutton *et al.,* 2009).

Various other methods adopted for the prolonged storage include cryopreservation (Drenth & Sendall, 2001), storage of cultures in mineral oil, where the *Phytophthora* is cultured on slopes made of rye agar and once the growth was established, sterile mineral oil was poured on top and stored. Vial containing sterile rye seeds immersed in sterile water was used for the mycelial growth in rye seed method and was stored. A modification to the above method involves the addition of mineral oil to the mycelium grown on rye seeds (Haichen *et al.,* 2018).

2.1.4. Identification of *Phytophthora* cultures

Identification of *Phytophthora* cultures purely based on morphology is radically insufficient as there is a chance of coincidence between the characters both intra and inter species (Anandaraj, 2012). Molecular techniques render simple and reliable outcomes, various methods commonly used for the species level identification includes RFLP (Restriction Fragment Length Polymorphism) analysis of Cox I and Cox II regions of mitochondrial DNA (Grünwald *et al.,* 2011; Martin and Tooley, 2004), nested PCR using internal transcribed spacer (ITS) region of rDNA (Bonants *et al.,* 1997). Besides these methods, translation elongation factor 1 alpha and beta tubulin sequences (Chávez-Ramírez *et al.,* 2021), recombinase polymerase amplification (RPA) and PCR based on gene *Ypt1* (Jeevalatha *et al.,* 2021), loop-mediated isothermal amplification (LAMP) with a species-specific quenching probe based on ITS region (Hieno *et al.,* 2020), recombinase polymerase amplification-lateral flow dipstick (RPA-LFD) method based on *Pcinn13739* gene (Chen *et al.,* 2022), multiplex PCR by target-specific primer extension with an array detection using Luminex xTAG based on ITS and Cox I (Kostov *et al.,* 2016), LAMP with ITS and Cox I primers (Hieno *et al.,* 2021), RPA and multiplex qPCR of *atp9* and *nad9* mitochondrial gene (Rojas *et al.,* 2017), *in planta* identification using peptide pattern (Berka *et al.,* 2020) and, hyperspectral imaging (Kool & Evenhuis, 2023) were used in identification of *Phytophthora* species.

2.2. Mating types

Both sexual and asexual reproduction can be seen in *P. capsici* and *P. tropicalis.* They are heterothallic in nature and require the presence of both the mating types

(A1 and A2) in the vicinity for the successful sexual mode of reproduction to occur. The process involves the differentiation of male and female gametangia after receiving the factors specific for mating, like α1 and α2 hormones (Barchenger *et al.,* 2018), which are acyclic oxygenated diterpenes synthesized from phytol. These hormones are universal as they can induce sexual reproduction in other species of *Phytophthora* as well (Tomura *et al.,* 2017). Phytol is the precursor for α2 hormone, α1 is synthesized from α2 hormone, which is being transported into the cells of A1 mating type (Lee *et al.,* 2012). The antagonistic activity of α2 was reported by Zhang *et al*. (2016), where they found that α1 induced sexual mode of reproduction in A2 was antagonized by the α2. Antheridia grow through oogonia, after meiosis, through the fertilization tube, the nuclei (haploid) reach oogonium resulting in the formation of amphigynous oospore (Lamour *et al.,* 2012) (Fig. 2.4). A1 and A2 mating types differ genotypically as the former is homozygous and the latter is heterozygous at the mating-type region on scaffold 4 of the reference genome (Quesada-Ocampo *et al.,* 2023).

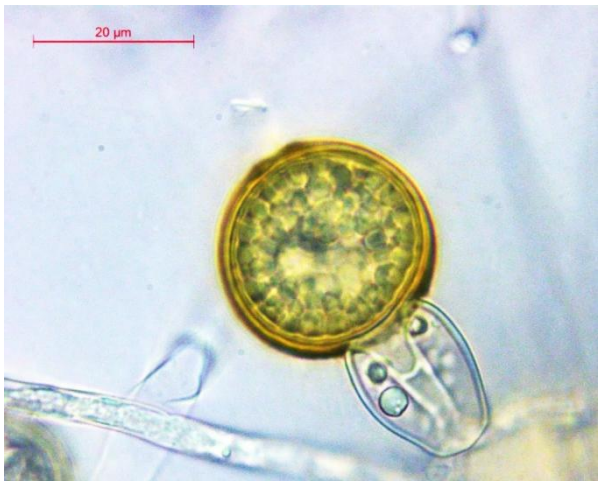


Fig. 2.4. Oospore produced by the fusion of A1 and A2 mating type.

The presence of A2 mating type apart from A1 mating type of *P. infestans* in Czech Republic was reported in 2006 (Mazáková *et al.,* 2006). The existence of both A1 and A2 mating type of *P. capsici* was reported in major black pepper growing regions in Vietnam (Truong *et al.,* 2010). In mating type studies from Pakistan (Raza *et al.,* 2021), the presence of both A1 and A2 mating types of *P. infestans* was

identified from the major potato-growing fields, and the A1 mating type was found to be the predominant one. The analysis of genotypes showed that the A2 mating type belongs to an aggressive lineage EU_13_A2 of the European population. The prevalence of both A1 and A2 mating type of *P. capsici* was reported in the infected soil samples from the chilli plantations of Bangladesh (Akter *et al.,* 2007). A report from Gautemala, a country in Central America confirmed the presence of A1 mating type of *P. capsici* through biological tests (Gonçalves-Pereira *et al.,* 2023). In China, A1 mating was predominant before 2012 and later, it turned out to be a self-fertile group of isolates in the Yunnan province (Tang *et al.,* 2023). However, there are not many reports available on the mating type studies in *P. tropicalis* except the present one.

2.2.1. Mating type markers

Various methods to distinguish the mating types have been reported. Mabon *et al*. (2021) designed a Multiplex PCR method where they targeted distinguishing *P. infestans* and A1 mating type in *P. infestans* population of France. Machine learning based approach was performed by Agho *et al*. (2024), where they analyzed SSR markers for mating type analysis and metalaxyl resistance by applying four approaches such as random forest, decision tree classifier, support vector machine and artificial neural network and found that random forest and support vector machine-based approach was most accurate when compared to rest of the approaches. The accurate determination of the mating type of the isolates is critical in population studies, irrespective of the ambiguities. Brylińska *et al*. (2018) validated different mating type-specific primers such as W16, S1 and PHYB. Their studies showed discrepancies in case of all the evaluated primers with that of the pairing test and it is highly recommended to validate the primers in local populations as it is highly dependent on the genotype of the pathogen. Mating type-specific primers were also developed in *P. capsici* infecting *Capsicum annum* using ISSR markers, specifically UBC821 (Li *et al.,* 2017)

2.3. Genome size estimation using flow cytometry

It is essential to unravel the complexities that dwell within the genome of the widely distributed and diverse pathogens like *Phytophthora,* which combats the changing environment and fungicide resistance (Cui *et al.,* 2019). Besides other pieces of evidence, knowledge on the genome size would provide information on species delimitation (Pflug *et al.,* 2020). Though flow cytometry has numerous applications, it is widely accepted for the accurate measurement of genome content. The fluorescent intensities of the particles stained with fluorochromes were measured as it passes through a mobile phase through the flow cell by hydrodynamic focusing. Here, the DNA content is measured relative to the reference standard. Types of standardization include external, pseudo-internal and internal standardization procedures. In external standardization, the nuclei of the unknown sample and the reference standard were analyzed separately. This is mainly used in the case of initial selection of appropriate reference standard or during ploidy studies. In pseudo-internal standardization, the nuclear suspension of the sample and the reference were prepared separately and then it was stained after mixing and were analyzed. As the results were not accurate, this is used only when it is difficult to co-process the sample and the reference simultaneously. Internal standardization is the most acceptable method for accurate analysis as the unknown and the reference were in the same environment throughout the process (Sliwinska *et al.,* 2022; Temsch *et al.,* 2021). Pflug *et al*. (2020) have compared the next-generation sequencing using k-mer methods to flow cytometry analysis and found that none of the estimations based on the sequence data were proved to be accurate in all the cases as the results were not consistent with the flow cytometry data and recommended the application of densitometry or flow cytometry in genome size estimation for validation.

In simple words, this technique works by calculating the relative fluorescence between the co-stained standard and the unknown samples (Hare *et al.,* 2011). This technique has a variety of applications in plant pathology, such as gene expression studies, determination of ploidy/genome size of the pathogen, detection of pathogen and its viability studies (D'Hondt *et al.,* 2011). The use of flow cytometry is

considered one of the standard techniques in plant genome size estimation; its application in studying the fungal genome size was infrequent. In light of this, Talhinhas *et al*. (2021) and Veselská *et al*. (2014) have standardized the protocol for fungal genome size estimation. Propidium iodide (PI) is one of the widely used fluorochromes which stains the majority of the sample and is easy to work with, it acts by intercalating at the DNA major groove. Llewellyn *et al*. (2023) performed flow cytometry analysis in *Ramalina farinacea,* a lichen-forming fungi, to ensure the completeness of the assembly sequenced by Oxford Nanopore long-read technology. There are only a limited number of reports available on genome size estimation of *Phytophthora* using flow cytometry. Van Poucke *et al*. (2021) characterized 27 of the hybrid *Phytophthora* species by sequencing and genome size estimation. The genome of two distinct isolates (genotypically as well as phenotypically) of *P. cinnamomi* was sequenced using Illumina sequencing and in addition, its genome size was estimated using flow cytometry (Shands *et al.,* 2024). Van Poucke *et al*. (2016) has determined the DNA content of *Phytophthora* isolates, which belong to 30 different species.

There are various factors which are to be taken care of when working with organisms with lower genome size such as its mass cultivation due to its small size; unistrain cultures at its active growth phase are preferred mostly. The presence of growth media would add to the background noise in the case of small cells; hence it is required to use high-specificity instruments. However, not all the buffers work with all the samples, therefore, it is necessary to standardize the buffer to be used. It was also recommended to use the lowest possible concentration of PI, and oftentimes, greater values of CVs were observed when working with smaller cells; therefore, criteria were kept lenient on acceptable accuracy (Čertnerová, 2022).

2.4. Pathogen distribution and Genetic variation

P. capsici was initially described from the United States (New Mexico) back in 1922 by L. H. Leonian as a causative organism of blight in peppers. In the 1920s, Tompkins and Tucker reported root rot of cucurbits from California (Café-Filho *et al.,* 1995), and in the 1930s, Bodine (1935) communicated from Colorado. Florida in

1931 (French-Monar *et al.,* 2006), 1933 in Arizona (Barchenger *et al.,* 2018), around 1930s in New York (Wiant & Tucker, 1940), during 1940s in Texas (Granke *et al.,* 2012), 1960s in New Jersey (Parra & Ristaino, 1998) and 1970s in Hawaii (Hunter *et al.,* 1971). *Phytophthora capsici* has been reported in many places ever since, like South Carolina, Michigan, Illinois and much more (Barchenger *et al.,* 2018).

Genetic variations are the amount of variations that are evident within a species in a population. Major forces that drive genetic variation include genetic drift, gene flow, recombination, mutation, hyphal anastomosis and parasexual events (Kumar & Verma, 2019), which result in the variation in the DNA sequence, protein profile, morphology and physiology of the organism. Genetic variation renders the ability to cope with stresses which are both biotic and abiotic in nature and also it preserves the beneficial alleles within a population (Salgotra *et al.,* 2023). The pathogen genotypes evolve over space and time and can acquire tolerance against the plants developed by resistance breeding (Dolatabadian & Fernando, 2022).

Lee *et al*. (2021) studied the genetic variation among the *P. capsici* isolates of Korea with different levels of aggressiveness and revealed the variations in genes associated with pathogenicity. Random amplified microsatellite (RAMS) studies showed that the *P. capsici* population infecting chilli from Bhutan was clonal (Rai *et al.,* 2020). Genetic diversity studies in *P. capsici* population of China using ISSR markers revealed the evolutionary potential of the pathogen (Li *et al.,* 2012). Chen *et al*. (2019) incorporated fluorescent labelling in SSR genotyping to investigate the genetic diversity of *P. capsici* isolated from various provinces of China, and clonal population was identified from only one of the provinces and sexual reproduction has a pivotal role in the genetic variation of the pathogen. A comparative study for detecting the genetic variation between sexually and asexually reproducing isolates of *P. capsici* from China using ISSR primers showed that though there exists genetic variation among the asexual population, the variation among the sexually reproducing population is greater (Li & Liu, 2021). Gobena *et al*. (2012) studied the genetic diversity of *P. capsici* infecting pumpkin from Argentina using AFLP markers and the population was identified as clonal. There are also a lot of reports

that address the diversity studies in *P. infestans* worldwide. A study from Canada on the genetic diversity of *P. infestans* revealed the presence of 25 new genotypes in addition to the existing 4 genotypes and also found that isolates of Pacific western Canada is more diverse than eastern Canada and were considerably resistant against metalaxyl-m (Babarinde *et al.,* 2024). The *P. infestans* population of the Northern Andean region and Venezuela were predominantly clonal and the emergence of *Avr3a* variants were shaped by migration (Cárdenas *et al.,* 2011). Shakya *et al*. (2018) reported the presence of greater genetic variation of *P. infestans* in the Michoacán area among the population of Michoacán, Mexico and Tlaxcala analyzed. The occurrence of *P. infestans* in China was associated with the human activities during the transportation of seed potato was revealed by phylogeographic studies (Gao *et al.,* 2020a). Variations linked to the aggressiveness of *P. infestans* was revealed recently by Alexander *et al*. (2024). Greater genetic variation was also observed in *P. palmivora* infecting cocoa plantations of Malaysia (Alsultan *et al.,* 2021).

2.4.2. Haplotype

Haplotype is the set of variations present at the polymorphic region of DNA which are uniparentally inherited (Dia & Cheeseman, 2021), hence, the chance of recombination is nil. It can also be explained as a set of variations in a gene with a strong linkage disequilibrium. There are mainly three ways to define haplotypes: one is by studying the haplotype diversity in a segment of chromosome, the next is by calculating linkage disequilibrium score and chance of recombination and the third method is by analyzing the SNPs (Bhat *et al.,* 2021). The parameters such as haplotype diversity and nucleotide diversity are considered as appropriate measures to explain the genetic diversity within a DNA sequence (Olou *et al.,* 2023).

Selecting mitochondrial genes for haplotype is considered a better option as they are maternally inherited without recombination and as the rate of mutation is uniform, tracing time of divergence is easier (Martin *et al.,* 2019). The rate of mutation is greater in the mitochondrial genome than in the nuclear genome due to the poor damage mechanism of DNA (Bi *et al.,* 2023). These mutations, which are either

SNPs or deletions, tend to have an impact on the phenotypic characters of the organism (Mossman *et al.,* 2019). However, nuclear gene-derived haplotypes do not provide much information on evolutionary background (Williams *et al.,* 2010). The biogenesis of mitochondria is dependent on both genomes viz., mitochondrial and nuclear and some of their regions were identified to be coevolved (Mossman *et al.,* 2019; Serrano *et al.,* 2024). These regions provide information on the difference in inheritance pattern, as the former is uniparentally and the latter is biparentally inherited (Mossman *et al.,* 2019). Small *et al*. (2004) have reported that the rate of evolution in nuclear gene is greater when compared to mitochondrial and organellar genes. The synonymous changes were also greater for nuclear genes. In nuclear genes, the promoter regions at the 5' untranslated region (UTR) have conserved domains; however, introns present here are occasionally variable. At non-synonymous sites of exons, the first and second codons remain conserved, whereas the third codon is variable. The 3' UTR region is also variable. The introns or UTRs have a lesser rate of evolution when compared to synonymous sites of exons. Various unlinked loci were used for the phylogenetic analysis independently and such information was often corroborated by the majority of data.

Quesada-Ocampo *et al*. (2011b) studied the genetic diversity of *P. capsici* infecting diverse hosts across various countries by analyzing the mitochondrial and nuclear haplotypes, which revealed the population structure of the pathogen across countries. The genetic structure of *P. capsici* isolates from irrigation water used in Michigan revealed that genetic variation identified between the isolates was less, and its presence is detected even after circulating the contaminated water to the non-host crops (Quesada-Ocampo *et al.,* 2011a). The mitochondrial DNA haplotype analysis from the herbarium specimens of the isolates collected during the Irish potato famine grouped the isolates into Ia, Ib, IIa and IIb and Ia was found to be associated with the disease. There are various theories proposed pertaining to the migration of *P. infestans*. One of them suggests that during the mid-19th century, *P. infestans* might have migrated to the United States from Mexico and Europe. The other theory suggests that it migrated from the Andean region to the United States

and Europe and yet another theory tells that from Mexico, it migrated to Peru and then to the United States and Europe (May & Ristaino, 2004).

Prathibha *et al*. (2024) revealed the existence of significant genetic diversity among the population of *P. meadii* infecting arecanut in India. By studying the eukaryotic translation elongation factor 1 alpha (eEF-1α), Wang *et al*. (2019) have established that among the potato and tomato infecting *P. infestans*, a greater genetic diversity was observed in those infecting tomato from Indian population and there is no gene flow between India and its neighbouring country China. The involvement of the United Kingdom (UK) and Europe population of *P. infestans* in late blight disease (2013-2014) was studied by Dey *et al*. (2018) from Northeastern Indian States and they also found that the pathogen is more aggressive in potato when compared with tomato and has acquired metalaxyl resistance. The *P. infestans* population of Himachal Pradesh were A2 mating type predominantly and shows moderate resistance to metalaxyl, and fall under Ia mtDNA haplotype (Sharma & Patil, 2016)

2.5. Immune system in plants

Due to the continuous selection pressures imposed by pathogens, the plant genome is actively involved in the coevolution process. This was later attributed to the gene-for-gene hypothesis (Flor, 1947), which states that the resistance gene (R gene) housed in the plant genome counteracts the corresponding avirulence gene (*Avr* gene) products like effectors of the pathogen. When a pathogen attacks a tolerant variety or a non-host, a hypersensitive response is initiated, where apoptosis occurs and ultimately restricts the accessibility of the pathogen to the uninfected tissues. The zigzag model, which explains the interaction between the pathogen and the host, was then proposed by Jones and Dangl (2006). This model explains two types of immune systems of the plant. The first is triggered by pathogen-associated molecular patterns or microbe-associated molecular patterns (PAMPS or MAMPS) (Pritchard & Birch, 2014). The pattern recognition receptors (PRRs) which are receptor-like proteins (RLPs) or receptor kinases (RKs) present on the plant cell recognize MAMP, PAMP or damage-associated molecular pattern (DAMP) results in inflammatory responses. RLPs have a ligand binding domain on the cell surface

and a transmembrane domain, whereas RKs have an additional kinase domain at the intracellular region for the downstream signalling. The second one is those triggered by certain factors called effectors, also known as effector-triggered immunity (ETI), which gets initiated immediately after the recognition of the *AVR* gene products by the R gene products and activates apoptosis as a localized immune response. The effector molecules are often perceived by the nucleotide-binding proteins with leucine repeat (NBS-LRR) (Zipfel, 2014). However, later it was noticed that pathogen secretes effector molecules which identified to suppress the PAMP triggered immunity (PTI) and establishes infection. To overcome ETI, the R gene products initiate immune responses like apoptosis to restrict the spread of infection, which was explained by the zigzag model (Keller *et al.,* 2016).

Based on the ligand binding domain, the PRRs are of different types, such as those with leucine-rich repeats (LRR) that bind flagellin, EF-Tu, an elongation factor or peptides like AtPep. Those with lysine motifs bind peptidoglycans or chitin present in bacteria and fungi, respectively. PRRs that are of lectin types bind lipopolysaccharides or exoplasmic ATP. Epidermal growth factor (EGF) containing PRRs bind oligogalacturonides derived from plants. The intracellular receptors, like NBS-LRR, bind to the effector molecules released by the pathogen and get activated to protect the target immune component. The systemic defense mechanism gets initiated following the activation of the PRR complex upon the appropriate ligand binding. The stress response exhibited by the plant cell includes an influx of calcium ions and reactive oxygen species will be produced at the exoplasmic region; this activates calcium-dependent protein kinase (CDPK) and mitogen-activated protein kinase (MAPK) pathway, hence implementing PTI (Couto & Zipfel, 2016).

Similar to *P. capsici*, *P. cinnamomi* is also a soil borne and hemi biotrophic in nature and it was reported by Midgley *et al.* (2024) that during the early biotrophic phase, the hypersensitive reaction like programmed cell death executed by the host plant gets suppressed by the effector molecule leading to the late necrotrophic phase where the effector molecule could activate the cell death.

2.5.1. Effectors

Effectors are the molecules secreted by the phytopathogen to attack the host plant. The avirulence gene that encodes for the effector molecule are present at the dynamic region of the genome. The sole purpose of the effector molecule is to suppress/overcome the immune response and initiate infection. However, as explained earlier, the host plants deploy counter mechanisms to restrict the influence of pathogen. This triggers a selection pressure on the effector molecule to prevent the recognition by PRRs or NBS-LRRs to establish infection, and this area of mechanism remains unknown (Boutemy *et al.,* 2011).

RxLR is a family of effector found in *Phytophthora* with a signal peptide domain for the secretion, a consensus RxLR (Arginine-any amino acid-Leucine-Arginine) sequence which is responsible for the translocation of protein into the plant cell, the region responsible for actual virulence (effector domain) located at the C-terminal and are highly diverse (Boutemy *et al.,* 2011; Rojas-Esteves *et al.,* 2020). It was hypothesized that modifications like insertion, deletion, duplication of tandem domain and replacement changes at alpha helix add up for the execution of virulence after eluding the recognition by PRRs (Boutemy *et al.,* 2011).

CRN (crinkling and necrosis) proteins are another class of effector which has motifs such as LXLFLAK and HVLVXXP at the N-terminal domain and a highly variable C-terminal domain. CRN effectors have an evident role in the immune suppression and causing death of the plant tissue (Midgley *et al.,* 2024). The localization studies in *P. capsici* by Stam *et al.* (2013) suggest that during infection, CRN effectors that activate cell death mainly target the nucleus of the host cell.

One of the methods which describes the mechanism by which *P. capsici* infects the host plant is by impairing EDS1 (Enhanced Disease Susceptibility 1), which plays an active role in the defense mechanism in plants by forming a heteromeric complex with PAD4. The RxLR effector, PcAvh103 of *P. capsici,* prevents the complex formation between EDS1 and PAD4, thereby suppressing the plant immunity (Li *et al.,* 2020).

RLCK-VII (Receptor-like cytoplasmic kinase subfamily VII) are the kinases in PRRs and were found to be impaired by the RxLR effector class of protein RxLR25 of *P. capsici,* thereby surpassing the MAMP-triggered immunity (Liang *et al.,* 2021).

2.5.1.1. Targets and mode of action of RxLR effectors

a) Transcription modification

Transcription factors are one of the common targets of the RxLR effector, as most of the cellular functions are controlled by the regulation of transcription. Zhu *et al*. (2023) studied the interaction of PsAvh113 of *P. sojae* with GmDPB, a transcription factor of *Glycine max,* and it was found that this interaction decreases the transcription of GmCAT1, which activates the immune-related genes due to the proteasomal degradation of GmDBP. Another RxLR effector of the same pathogen PsAvh110, hinders the immune pathway by preventing the transcriptional complex formation between GmLHP1-2, a heterochromatin protein and GmPHD6, a homeodomain finger protein (Qiu *et al.,* 2023).

One of the RxLR effector HaRxLL470 of *Hyaloperonospora arabidopsis,* an oomycete, was reported to interact with the HY5 protein of *Arabidopsis thaliana*, which is involved in photomorphogenesis and in the regulation of defense-related genes, thereby compromising the immunity of the host (Chen *et al.,* 2021a).

The role of PvRxLR111 effector of *Plasmopara viticola* in establishing infection in *Nicotiana benthamiana* was described by Ma *et al*. (2021). The effector PvRxLR111 interacts with a transcription factor VvWRKY40, which inhibits flg22 (Bacterial flagellin peptide) induced immunity, i.e., VvWRKY40 acts as a negative regulator of host immunity. Another example of negative regulation is the interaction between RxLR effector Pi22798 of *P. infestans* and StKNOX3 transcription factor of potato, which will enhance its expression, hence making the host more susceptible to infection (Zhou *et al.,* 2022).

b) Phosphate modification

The overexpression of *P. capsici* RxLR effector *PcAvh1* enhances the disease progression in *N. benthamiana,* which acts by interfering with protein phosphatase 2A (PP2Aa) involved in the immune regulation (Chen *et al.,* 2019). RxLR effector molecules secreted by *P. infestans,* such as PITG20303 and PITG20300, slip away the recognition by Rpi-blb2 resistance protein and stabilize StMKK1, the mitogen-activated protein kinase (MAPK) protein that acts as a negative regulator of plant immunity. This suppresses the PAMP-triggered immunity (Du *et al.,* 2021). The avirulence gene of *Plasmodiophora brassicae* encodes for an RxLR effector, PBZF1 which targets SNF1-related kinase 1 (SnRK1) of *Arabidopsis thaliana* and inhibits SnRK1.1, which has an active role in shifting the utilization of energy source from growth-related process to activation of defense-related pathway (Chen *et al.,* 2021b).

c) Immune regulators manipulation

Immune regulators are the molecules that direct the host immune response against the pathogen. RxLR242 of *P. capsici* interacts with RAB proteins which are the GTPase family of proteins which plays role in regulating membrane trafficking for instance, RxLR242 interacts with RABE1-7 hinders the apoplastic secretion of PATHOGENESIS RELATED 1 (PIR1), and when RxLR242 interacts with RABA4-3, FLAGELLIN SENSING 2 trafficking gets disrupted (Li *et al.,* 2022). Avh94, an RxLR effector of *P. sojae* targets and hinders the jasmonic acid signalling by stabilizing its repressor JAZ1/2 (Zhao *et al.,* 2022). Avh241 interfere with the self-association of NDR1 (non-race specific disease resistance 1), affecting the NDR1 mediated ETI responses (Yang *et al.,* 2021).

d) Alteration of cellular trafficking

One of the mechanisms adopted by plants to kill the pathogen is by releasing the toxic antimicrobials, this is regulated by membrane trafficking as it requires energy and is toxic to plant cells as well (Yun & Kwon, 2017). The RxLR242 of *P. capsici* interacts with RABE1-7 and prevents it from associating with vesicle-related proteins, hence interfering with the trafficking (Li *et al.,* 2022). The RxLR242 also

interacts with RABA4-3 and silences it; this inhibits the endoplasmic reticulum to plasma membrane trafficking of FLS2, which is a pattern recognition receptor that recognizes bacteria PAMP flagellin 22 (flg22) (Li *et al.,* 2022). Another RxLR effector PsAvh181 of *P. sojae* targets soybean N-ethylmaleimide-sensitive factor (NSF) attachment protein GmSNAP-1, hence hindering the association of GmNSF of SNARE and GmSNAP-1 thus manipulating the trafficking (Wang *et al.,* 2021). Autophagy is yet another defense mechanism exhibited by the host, i.e., during infection, autophagosome with ATG8CL/Joka2 gets accumulated at the perimicrobial membrane to restrict the pathogen entry into the host cell. One of the RxLR effectors of *P. infestans* PexRD54 acts as a competitive inhibitor of autophagy cargo receptor Joka2 at the extra haustorial membrane and prevents autophagy (Dagdas *et al.,* 2018).

e) Alteration in ubiquitination

The immune response execution not only requires the regulated synthesis of proteins but also its regulated degradation, which occurs through the ubiquitin-proteasome system (Langin *et al.,* 2023). Avr1d of *P. sojae* binds competitively with GmPUB13, an E3 ubiquitin ligase of soybean and inhibits it and establishes the infection (Lin *et al.,* 2021). *P. infestans* RxLR effector Pi06432 alter the salicylic acid-mediated immune responses in potato by silencing StUDP (ubiquitin-like domain-containing proteins). StUDP normally destabilizes the 26S proteasome subunit StRPT3b (regulatory particle triple-A ATPase) and disrupt its activity and also it destabilizes SA biosynthesis transcription factor SARD1 (systemic acquired resistance deficient 1) (Wang *et al.,* 2023). Avr8 of *P. infestans* destabilize StDeSI2 (desumoylating isopeptidase) by 26S proteasome and disrupts the pattern-triggered immune response (Jiang *et al.,* 2023). *Plasmopara viticola* promotes its infection in grapevine via RxLR50253, which is found to interact with VpBPA1 of the host and favors the colonization and reduce H_2O_2 accumulation during infection (Yin *et al.,* 2022).

f) Manipulation of chloroplast-mediated immunity

Chloroplasts act in defense response by generating Reactive Oxygen Species (ROS) and also produce defense-related phytohormones (Nomura *et al.,* 2012). The

transportation of TalSP (a chloroplast protein) to the chloroplast was hampered by two of the effectors of *Puccinia striiformis* f. sp. *tritici*, like Pst_4 and Pst_5, leading to decreased ROS accumulation and immunity. Similarly, Pst_12806 interacts with TalSP and disrupts the electron transport (Breen *et al.,* 2022). The nucleotide binding leucine rich repeat, Rpi-vnt1.1 of the host, associates with a nuclear encoded full length chloroplast proteins GLYK (glycerate 3-kinase) in the presence of light and act against a *P. infestans* effector AVRvnt1. In the case of plants that lack this NLR, it prevents the association of Rpi-vnt1.1 with full-length GLYK, hence compromising the immune response (Gao *et al.,* 2020b). A chloroplast protein, VpPsbP, is targeted by *Plasmopara viticola* and increases the susceptibility of grapevine by activating O2-mediated signalling and reducing H_2O_2 accumulation (Liu *et al.,* 2021).

g) Post-translational modification of target proteins

Post-translational modifications play an active role in the regulation of plant immune responses (Gough & Sadanandom, 2021). The RxLR effector CRISIS2 (cell death inducer suppressing immune system) of *P. capsici* attacks the plant cell by binding with plasma membrane H^+-ATPase (PMA) and inactivating it, causing the alkalization of the host cell apoplast (Seo *et al.,* 2023). An RxLR effector FIRE of *P. palmivora* interacts with 14-3-3 proteins when it is phosphorylated and during infection; both get relocalized at the haustoria, increasing the susceptibility of the host (Evangelisti *et al.,* 2023).

CHAPTER 3

SPECIES DISTRIBUTION AND MATING TYPE ANALYSIS OF *PHYTOPHTHORA* SPECIES INFECTING BLACK PEPPER

Abstract

The foot rot caused by *Phytophthora* is one of the major concerns in the scenario of global black pepper cultivation. The disease can pose a major threat if left unnoticed due to its rapid spread across the field. Various species of *Phytophthora* associated with black pepper diseases have been identified hitherto. Identification and timely action against the disease helps in controlling and spread of the disease. In the present study, a total of 164 *Phytophthora* isolates obtained from black pepper growing areas in Kerala, Karnataka, Tamil Nadu and Goa were analyzed by PCR assay using Ypt1 gene-based species-specific and ITS primers for species identification. The results showed that two species, *P. capsici* and *P. tropicalis* were almost equal in numbers. Few isolates belonged to some other species such as *P. meadii* (5 isolates), *P. nicotianae* (3 isolates) and *P. palmivora* (1 isolate), infecting other component crops of black pepper ecosystem. Mating type of the isolates were determined by pairing them with reference A1 and A2 mating type isolates. All the isolates produced oospores with A2 reference culture which indicated that they all belong to A1 mating type. An attempt was also made to develop mating type specific marker using UBC and ISSR primers for the robust and rapid detection of the mating type. But markers could not be developed and possible reason would be A2 mating culture of *P. capsici* isolate of *Capsicum annuum* was used due to the non-availability A2 mating type culture of black pepper *P. capsici* isolate. The mating type analysis also gave us a basic idea regarding the diversity, as there is a decreased chance of sexual reproduction occurring among black pepper isolates since A1 was the only mating type detected so far. The study also gave an insight into the abundance of each species in different geographic locations.

3.1. Introduction

Foot rot caused by *Phytophthora* is a serious matter of concern as the infection is often detrimental to the black pepper plants. In India, black pepper is mainly grown in South Indian states like Karnataka, Kerala, Tamil Nadu and also grown in parts of Goa and Maharashtra. Black pepper is a crop of immense trade value, which entails considerable benefits within. One of the impediments to its cultivation is the foot rot disease caused by *Phytophthora*. *Phytophthora* is regarded as the fifth most destructive pathogen as it rapidly spreads and causes immense economic loss. Its occurrence was first reported in New Mexico back in 1922 and incidents affecting pepper were reported in countries like Tunisia, Brazil, South Africa, Bulgaria, Korea and Netherlands (Moreira-Morrillo *et al.,* 2023). Subsequent occurrences have been reported in countries like Indonesia (Erwin & Ribeiro, 1996), Spain (Silvar *et al.,* 2006) China (Liu, 2008), Vietnam (Long, 2015), Malaysia (Farhana *et al.,* 2013) and many more.

The foot rot disease has prevailed in India since 1902, and the causative organism was identified much later in 1966 (Sarma & Anandaraj, 1997). The *Phytophthora* species infecting black pepper include *P. capsici, P. tropicalis* (Bhai *et al.,* 2022), *P. nicotianae* (Roy *et al.,* 2009), *P. palmivora, P. parasitica* and *P. citrophthora* (Anandaraj, 2012). In a study conducted by Tsao and Alizadeh in 1988, they compared the morphological distinction between *P. capsici* and '*P. palmivora* MF4' and discovered a clear distinction between the two groups of isolates. Later in 2001, Aragaki and Uchida proposed *P. tropicalis* taxon based on morphological and pathological studies. *P. capsici* and *P. tropicalis* (grouped initially in clade 4) were grouped in clade 2b among the 10 phylogenetic clades of *Phytophthora* species. There has always been a dilemma regarding the distinction of the two species, as there were similarities in their morphology. However, studies showed that they are genetically distinct, and the evidence also showed that *P. capsici* radiated from *P. tropicalis* (Quesada-Ocampo *et al.,* 2023).

Phytophthora is a soil-borne pathogen, and it produces both sexual and asexual spores, which serve both as a resting structure and as a propagule for the disease

progression. *Phytophthora* produces sporangia asexually, which are caducous and papillate and release motile biflagellate zoospores (Sanogo, 2007), which swim readily in water and land on soil or the plant surface where they encyst and initiate the infection. Sexual reproduction occurs as both *P. capsici* and *P. tropicalis* are heterothallic species; essentially, both A1 and A2 mating types must be present in the vicinity for the sexual mode of reproduction to occur. Mating occurs by the production of mating hormones, which induce gametangia formation. Male gametangia are called antheridium, which fuses with a female counterpart, which is called oogonium which finally produces oospores (Ristaino *et al.,* 1999), which can remain viable in the soil for up to three years (Babadoost *et al.,* 2013). In India, no A2 mating type of *P. capsici* and *P. tropicalis* isolates infecting black pepper have been reported to date. However, both A1 and A2 mating types of *P. capsici* infecting cocoa were reported and are capable of producing oospores inter and intra-specifically (Chowdappa & Chandramohanan, 2002).

The disease diagnostics and the identification of the causative organism associated with the disease are often crucial for taking actions toward its control, management and in the successful deployment of preventive measures to ensure better plant health. There are reports which show the occurrence of *Phytophthora* species other than *P. capsici* and *P. tropicalis* associated with the disease, for example, the existence of *P. nicotianae* causing foot rot in black pepper in Vietnam (Thao *et al.,* 2024). The chance of recurrent occurrences in a field has to be taken care of beforehand, as the *Phytophthora* produces survival structures which act on favourable environmental conditions. Hence, in the present study, the species of *Phytophthora* associated with foot rot disease of black pepper, its abundance and distribution and the mating type of the isolates were investigated across the major black pepper growing regions of India.

3.2. Materials and methods

3.2.1. Source of isolates and maintenance

The *Phytophthora* isolates, which were collected from various black pepper growing areas over the period of 1997 to 2018, were obtained from the National Repository

of *Phytophthora,* ICAR-IISR, Kozhikode as depicted in Fig. 3.1 and are listed in Table 3.1. The mycelial discs of 5 mm were inoculated onto the surface of carrot agar medium (Brasier, 1967) and incubated at 25±1 °C. For long-term storage, the mycelial discs were stored at 25± 1 °C in a vial containing sterile distilled water.

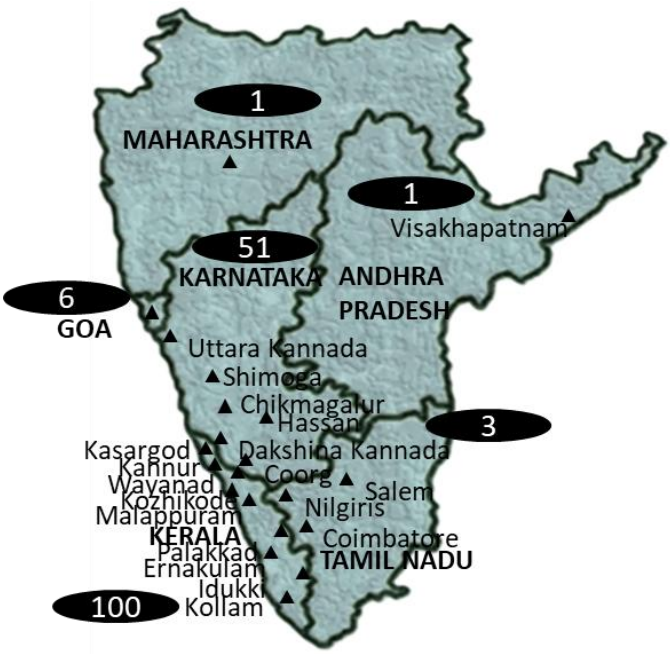


Fig. 3.1. Map of South India showing the total number of isolates and places from where *Phytophthora* isolates were collected from infected black pepper

Table 3.1. Details of the isolates used to study the species identity

Sl. No.	Isolate	Place of collection	State
1	97-11	Pulpally	Kerala
2	97-19	Kottakkal	Kerala
3	97-52	Pulpally	Kerala
4	97-53	Pulpally	Kerala
5	97-54	Ayoor	Kerala
6	97-55	Valparai	Tamil Nadu
7	98-02	Peruvannamuzhi	Kerala
8	98-03	Peruvannamuzhi	Kerala
9	98-07	Sakleshpur	Karnataka
10	98-60	Valnoor, Kodagu	Karnataka
11	98-70	Vageri, Wayanad	Kerala

12	98-76	Sundicopa, Kodagu	Karnataka
13	98-17	Pulpally	Kerala
14	98-59	Chettalli CRSS farm	Karnataka
15	98-66	Valnoor, Kodagu	Karnataka
16	98-65	Valnoor, Kodagu	Karnataka
17	98-92	Sundicopa	Karnataka
18	98-93	Adyanadka	Karnataka
19	98-71	Sundicopa, Kodagu	Karnataka
20	98-128	Kunnamangalam	Kerala
21	98-135	Theli, Sirsi	Karnataka
22	98-143	Yadally, Sirsi	Karnataka
23	98-145	Puttanamana	Karnataka
24	98-156	Sirsi	Karnataka
25	98-157	Sirsi	Karnataka
26	98-171	Adyanadka, Putur	Karnataka
27	98-172	Isloor, Sirsi	Karnataka
28	98-175	Isloor, Sirsi	Karnataka
29	98-177	Rayarpet	Karnataka
30	98-182	Isloor, Sirsi	Karnataka
31	98-184	Shelloor	Karnataka
32	99-91	Thamarasseri	Kerala
33	99-124	Gudalur	Tamil Nadu
34	99-136	Silver Cloud, Wayanad	Kerala
35	99-139	Meenangadi	Kerala
36	99-144	Pollibetta	Karnataka
37	99-145	Suganthagiri	Kerala
38	99-166	Peruvannamuzhi	Kerala
39	00-38	Sidapur	Karnataka
40	01-20	Silent Valley, Palakkad	Kerala
41	03-07	Adivaram	Kerala
42	05-17	Kalarickal Plantation, Chettalli	Karnataka
43	06-10	Iritti (Edayannur), Kannur	Kerala
44	06-11	Iritti (Edayannur), Kannur	Kerala
45	06-17	Rasi Estate, Yercaud	Tamil Nadu
46	08-07	Koorachundu	Kerala
47	09-01	Peruvannamuzhi	Kerala
48	09-08	Coorg, Karnataka	Karnataka
49	09-11	Peruvannamuzhi farm	Kerala
50	09-13	Eriapally, Pulpally	Kerala

51	09-18	Pamathatt, Kasargod	Kerala
52	09-19	Therthalli, Kannur	Kerala
53	09-25	Kanjirapuzha	Kerala
54	09-26	Aathur Cross, Kodagu	Karnataka
55	09-36	Mudigere	Karnataka
56	09-39	Mudigere	Karnataka
57	11-14	Kargunda	Karnataka
58	11-19	Sannapulikot	Karnataka
59	11-20	Sannapulikot	Karnataka
60	11-21	Ayyangeri	Karnataka
61	11-28	Wayanad	Kerala
62	11-29	Wayanad	Kerala
63	11-30	Wayanad	Kerala
64	11-32	Wayanad	Kerala
65	13-07	Kattikulam, Mananthavadi	Kerala
66	13-09	Kattikulam, 2nd gate	Kerala
67	13-10	Mananthavadi	Kerala
68	13-11	Cheloor, Mananthavadi	Kerala
69	13-12	Kurukanmoola, Mananthavadi	Kerala
70	13-23	Nedumkandam, Idukki	Kerala
71	13-24	Nedumkandam, Idukki	Kerala
72	13-33	Kumali, Idukki	Kerala
73	13-34	Vandiperiyar, Idukki	Kerala
74	13-36	Peermade, Idukki	Kerala
75	13-37	Erattayar, Idukki	Kerala
76	13-45	Pilikod	Kerala
77	13-49	Goa	Goa
78	13-51	Goa	Goa
79	13-52	Goa	Goa
80	13-53	Goa	Goa
81	98-48	Asoka Plantations, Madikeri	Karnataka
82	98-81	Malappuram, Kalpakancherry	Kerala
83	98-87	Kunnamangalam	Kerala
84	98-142	Nukkal, Sirsi	Karnataka
85	98-155	Chettalli	Karnataka
86	98-164	Kangode, Sirsi	Karnataka
87	98-174	Isloor, Sirsi	Karnataka
88	98-176	Sirsi	Karnataka
89	98-185	Puttanamana, Sirsi	Karnataka

90	98-183	Yadally	Karnataka
91	98-198	Manjachola	Kerala
92	99-132	Wayanad	Kerala
93	00-42	Sirsi	Karnataka
94	01-04	Adivaram	Kerala
95	02-20	Silent Vally, Palakkad	Kerala
96	02-02	Wayanad	Kerala
97	03-02	Vythiri	Kerala
98	03-10	Thikkodi	Kerala
99	05-06	Peruvannamuzhi	Kerala
100	05-09	Chelavoor	Kerala
101	05-13	Horticulture farm Appangala	Karnataka
102	05-14	Horticulture farm Appangala	Karnataka
103	05-19	Horticulture farm, Chettalli	Karnataka
104	06-01	Koothali farm	Kerala
105	06-02	Koothali farm	Kerala
106	06-03	IISR- Nursery	Kerala
107	06-04	Puthupadi Nursery	Kerala
108	06-09	Pepper shed IISR campus	Kerala
109	06-12	Thiruvampadi	Kerala
110	06-13	Iritti (Edayannur), Kannur	Kerala
111	07-02	Peruvannamuzhi	Kerala
112	07-05	IISR campus Nursery shed	Kerala
113	07-06	Wayanad	Kerala
114	07-07	Wayanad	Kerala
115	08-01	Peruvannamuzhi	Kerala
116	08-02	Thamarassery	Kerala
117	08-03	Peruvannamuzhi farm	Kerala
118	08-05	Koppa	Karnataka
119	09-02	Peruvannamuzhi	Kerala
120	09-03	Peruvannamuzhi, Kozhikode	Kerala
121	09-15	Vemam, Wyanad	Kerala
122	10-02	Bommanahally, Sakleshpur	Karnataka
123	11-11	IISR campus, Chelavoor	Kerala
124	11-13	Kargunda	Karnataka
125	11-16	Cherambane	Karnataka
126	11-17	Koopatty	Karnataka
127	13-17	Meppadi	Kerala
128	13-21	Adimali, Idukki	Kerala

129	13-29	Pampadumpara, Idukki	Kerala
130	13-30	Puttadi, Idukki	Kerala
131	13-40	Kalluvalappu	Kerala
132	13-41	Madikai	Kerala
133	13-42	Madikai	Kerala
134	13-46	Goa	Goa
135	13-48	Goa	Goa
136	18-01	Plot 24, Kozhikode	Kerala
137	18-02	Plot 1, Kozhikode	Kerala
138	18-09	Plot 32	Kerala
139	18-11	Plot 29	Kerala
140	18-12	Edavanna	Kerala
141	18-13	Idukki	Kerala
142	18-20	Wayanad	Kerala
143	20-01	IISR, Kozhikode	Kerala
144	20-02	Peruvannamuzhi	Kerala
145	20-03	IISR, Kozhikode	Kerala
146	20-04	Mudigere	Karnataka
147	20-05	Panniyur, Kannur	Kerala
148	20-06	Ambalavayal, Wayanad	Kerala
149	20-07	Panniyur, Kannur	Kerala
150	21-01	Peruvannamuzhi	Kerala
151	21-02	Panniyur, Kannur	Kerala
152	22-01	Peruvannamuzhi	Kerala
153	22-02	Peruvannamuzhi	Kerala
154	22-03	Maharashtra	Maharashtra
155	23-01	Peruvannamuzhi	Kerala
156	99-188	Gunalanka	Andhra Pradesh
157	09-03	Peruvannamuzhi	Kerala
158	09-10	Kalpatta	Kerala
159	09-22	Payamukk, Kannur	Kerala
160	09-27	Makkandur, Coorg	Karnataka
161	13-38	Chittariparambu	Kerala
162	98-95	Adivaram	Kerala
163	02-17	Silent Valley	Kerala
164	98-186	Balagatte	Karnataka

3.2.2. Analysis of species distribution pattern

Species identification was performed using Polymerase Chain Reaction (PCR), consisting of 20 µL reaction per tube. Composition of reaction mix includes 2 µL of Taq Buffer A (Tris with 15mM $MgCl_2$, GeNei^{TM}) 0.5 µL of 2mM dNTP Mix (Thermo Scientific), 0.5 µL of 10 µM each of forward and reverse primers (Table 3.2), 0.3 µL of Taq DNA Polymerase (1 U/ µL, GeNei^{TM}) and 1 µL of 100ng/µL of template DNA. The temperature cycle consisted of initial denaturation at 94^{o}C for 5 min followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 60^{o}C for 30 sec, extension at 72^{o}C for 45 sec, and final extension for 10 min. The PCR assay was performed using Applied Biosystems^{TM} ProFlex^{TM} PCR System. The PCR products were separated in 2% agarose gel immersed in 1x TAE buffer at 90 volts for 2 hours. The products were visualized and photographed using UV trans-illuminator (G: Box Chemi XR5, Syngene).

Table 3.2. List of primers used for species identification

Sl. No.	Primer	Sequence	Amplicon size (bp)
1	PC-Y-FP	5'-AAGCAACCAAAGTTCAAGACGTTT-3'	302
2	PT-Y-FP	5'-GATTGTAAGCACTTGTTATCCATC-3'	301
	COM-Y-RP2	5'-GCAGGCGTATCTGAAATTTGC-3'	
3	PP-F	5'-TGCTGGCGGCTGCTGTTGGGA-3'	320
4	PN-F	5'-CTGCTGAGTGAGCCCTATCAAAAAAAA-3'	320
5	PMPCO-F	5'-GCTTGGCATTTTGCTGAGCCGC-3'	320
	COM-R	5'-CCACCGACTACACGGAAGGAAGAA-3'	

3.2.3. Mating type analysis (pairing test)

Pairing test was performed with three-day-old cultures grown on carrot agar medium. To identify the mating type, the *Phytophthora* isolates of unknown mating type were paired with the reference *P. capsici* and *P. tropicalis* isolates obtained from the American Type Culture Collection, Manassas, USA (www.atcc.org). A five

mm disc *of Phytophthora* of unknown mating type was inoculated on 10% clarified V8 agar, and the disc of reference isolate either A1 isolate (ATCC-MYA-2338, ATCC-MYA-76651, ATCC-MYA-52239) or A2 isolate (ATCC-MYA-4034) was inoculated on the opposite side at a distance of 3 cm apart. The pairing test was performed in triplicate. The plate was incubated in the dark at 25°C for about a month and was observed under the Leica DM 5000 B microscope (Leica Mikrosystems Vertrich GmbH, Germany) for the production of oospores. Those isolates which produced oospore with A1 was recorded as A2 mating type and vice versa.

3.2.4. Development of PCR markers for mating type identification

3.2.4.1. DNA extraction and PCR with ISSR primers

DNA extraction from both A1 (PC05-06) and A2 mating type (ATCC-MYA-4034) of *P. capsici* was carried out according to Sheji *et al.* (2009) with slight modifications (Jeevalatha *et al.,* 2021). Agar plugs from 3-day-old cultures were inoculated into Ribeiro's broth (Erwin & Ribeiro 1996), and actively growing mycelia were harvested on the 4th day and blot-dried on sterile filter paper. The tissue was macerated using a sterile mortar and pestle with 1 mL of STE extraction buffer and glass wool. The debris was removed by centrifuging at 12,000 rpm for 10 min. 500 µL of tris phenol, chloroform, isoamyl alcohol (25:24:1) was mixed with the supernatant by gentle inversion and centrifuged at 12,000 rpm for 10 min. The tubes were centrifuged at 12,000 rpm for 10 min and to the aqueous layer, an equal volume of chloroform, isoamyl alcohol (24:1) was added and mixed by gentle inversion. Aqueous layer was collected by centrifuging at 12,000 rpm for 10 min, and 0.6 volume of isopropanol was added and mixed by gentle inversion. The pellet was obtained by centrifuging at 12,000 rpm for 10 min, and then it was washed with 70% ethanol. The pellet was then dissolved in 30 µL of nuclease-free water and incubated at 37°C for RNase treatment. The quality of the DNA was checked using DS-11+ Spectrophotometer (DeNovix®).

A total of 51 ISSR markers listed in Table 3.3 were used for the study. PCR amplification was carried out in a 20 µL reaction volume containing 10 µL of

Emerald Amp GT PCR Master Mix (2X Premix), 0.25 µM of primer and 5ng of DNA. Thermal cycling was performed in Gradient Thermal Cycler IG-96GEP by following the thermal cycling of an initial denaturation of 10 min at 95°C followed by 35 cycles of denaturation at 95°C for 1 min, annealing for 1 min, extension at 72°C for 2 min, and final extension of 72°C for 10 min. The amplicons were run in 1% agarose gel in 1X TAE buffer at 100 V for 2 hours and visualized under UV transilluminator (G: Box Chemi XR5, Syngene). The unique bands that differentiate the two mating types (Fig. 3.2) were cut out and eluted using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany) by following the manufacturer's instructions. The quality of the eluted products was checked using agarose gel electrophoresis and DS-11+ Spectrophotometer (DeNovix®).

Table 3.3. List of ISSR primers used for the study

Sl. No	Primer	Sequence	Annealing temperature (°C)
1	ISSR4	5'-GTGTGTGTGTGTAY-3'	45
2	ISSR12	5'-CACCACCACGC-3'	45
3	ISSR13	5'-AGTGAGTGAGTGGG-3'	44
4	ISSR17	5'-CACACACACACAAG-3'	45
5	$T_3(ATT)_4$	5'-TTTATTATTATTATT-3'	50
6	$T(GA)_8$	5'-TGAGAGAGAGAGAGAGA-3'	50
7	$TC(ATT)_4$	5'-TCATTATTATTATT-3'	50
8	$(TG)_8AGT$	5'-TGTGTGTGTGTGTGTGAGT-3'	52
9	$(TG)_7$	5'-TGTGTGTGTGTGTG-3'	50
10	UBC-806	5'-TATATATATATATATAG-3'	31
11	UBC-808	5'-AGAGAGAGAGAGAGAGC-3'	46
12	UBC-809	5'-AGAGAGAGAGAGAGAGG-3'	60
13	UBC-811	5'-GAGAGAGAGAGAGAGAC-3'	45
14	UBC-813	5'-CTCTCTCTCTCTCTCTT-3'	50
15	UBC-814	5'-CTCTCTCTCTCTCTCTA-3'	46
16	UBC-815	5'-CTCTCTCTCTCTCTCTG-3'	44
17	UBC-816	5'-CACACACACACACACAT-3'	58
18	UBC-817	5'-CACACACACACACACAA-3'	45

19	UBC-818	5’-CACACACACACACACAG-3’	50
20	UBC-820	5’-GTGTGTGTGTGTGTGTC-3’	50
21	UBC-821	5’-GTGTGTGTGTGTGTGTT-3’	56
22	UBC-823	5’-TCTCTCTCTCTCTCTCC-3’	54
23	UBC-824	5’-TCTCTCTCTCTCTCTCG-3’	49
24	UBC-825	5’-ACACACACACACACACT-3’	46
25	UBC-826	5’-ACACACACACACACACC-3’	53
26	UBC-827	5’-ACACACACACACACACG-3’	54
27	UBC-828	5’-TGTGTGTGTGTGTGTGA-3’	50
28	UBC-829	5’-TGTGTGTGTGTGTGTGC-3’	50
29	UBC-830	5’-TGTGTGTGTGTGTGTGG-3’	50
30	UBC-835	5’-AGAGAGAGAGAGAGAGYC-3’	42
31	UBC-836	5’-AGAGAGAGAGAGAGAGCA-3’	44
32	UBC-841	5’-GAGAGAGAGAGAGAGAYC-3’	48
33	UBC-843	5’-CTCTCTCTCTCTCTCTRA-3’	46
34	UBC-844	5’-AGAGAGAGAGAGAGAGYT-3’	48
35	UBC-845	5’-CTCTCTCTCTCTCTCTRG-3’	48
36	UBC-846	5’-CACACACACACACACART-3’	48
37	UBC-850	5’-GTGTGTGTGTGTGTGTYC-3’	53
38	UBC-851	5’-GTGTGTGTGTGTGTGTYG-3’	53
39	UBC-852	5’-ACACACACACACACACT-3’	48
40	UBC-855	5’-ACACACACACACACACYT-3’	56
41	UBC-856	5’-ACACACACACACACACYA-3’	53
42	UBC-857	5’-ACACACACACACACACTG-3’	54
43	UBC-858	5’-TGTGTGTGTGTGTGTGRT-3’	53
44	UBC-860	5’-TGTGTGTGTGTGTGTGRA-3’	52
45	UBC-865	5’- CCGCCGCCGCCGCCGCCG	47
46	UBC-866	5’-CTCCTCCTCCTCCTCCTC-3’	50
47	UBC-867	5’-GGCGGCGGCGGCGGCGGC-3’	56
48	UBC-884	5’-ACTGCTGAGAGAGAGAGAGA-3’	50
49	UBC-891	5’-TGTGTGTGTGTGTGHVH-3’	52
50	UBC-896	5’-AGGTCGCGGCCGCNNNNNNATG-3’	50
51	UBC-897	5’CCGACTCGAGNNNNNNATGTGG-3’	50

3.2.4.2 Cloning, transformation and validation of developed primers

Unique size bands specific to A1 and A2 mating types were observed in five primers, and they are listed in Table 3.4. These bands were cloned using Thermo Scientific CloneJET PCR Cloning Kit, by following the manufacturer's instructions. Transformation was performed using TransformAid Bacterial Transformation Kit (Thermo Scientific™) by following the manufacturer's instructions. The cells were then plated onto an LB agar plate supplemented with Ampicillin, X-Gal and IPTG and incubated overnight at 37°C. The white colonies were screened for the insert by colony PCR. The colonies with insert were inoculated in 5 mL of LB broth supplemented with Ampicillin and incubated overnight at 37°C. Plasmid isolation was performed using NucleoSpin Plasmid Mini kit for plasmid DNA (Macherey-Nagel, Germany) by following the manufacturer's instructions. The presence of the insert was further confirmed by performing PCR by following the conditions mentioned above. The plasmid was sent for sequencing at Eurofins Genomics Pvt. Ltd, Bangalore, which were then used for designing the primers (Table 3.5).

The developed primers were validated by PCR. 20 µL of PCR reaction mix was prepared, which consists of 10 µL of Emerald Amp GT PCR Master Mix (2X Premix), 0.5 µL each of forward and reverse primers and 1 µL of 100 ng/µL DNA and the total volume was made up using nuclease-free water. The PCR was carried out in Applied Biosystems™ ProFlex™ PCR System by following the temperature profile given in Section 3.2.4.1, and the annealing temperature was 50°C for UBC850-A1-FP/UBC850-A1-RP and 58°C for IS4-FP/IS4-RP. The products were run in 1% agarose gel.

Table 3.4. List of unique bands selected for cloning to develop mating type marker

Primer	Isolate	Size of amplicon cloned
UBC850	05-06 (A1)	1300 bp
	4034 (A2)	750 bp
UBC851	05-06 (A1)	1500 bp
		850 bp
	4034 (A2)	1850 bp
		1600 bp
ISSR4	05-06 (A1)	1100 bp
	4034 (A2)	1200 bp
$(TG)_7$	4034 (A2)	1400 bp
$(GA)_8C$	4034 (A2)	1600 bp

Table 3.5. Primers designed from ISSR4 and UBC850

Primer	Sequence	Length of amplicon (bp)
IS4- FP	5' -ACACACACACACACACCTCAACGGA-3'	1134
IS4- RP	5' -ACACACACACACACACCCAACTCTG-3'	
UBC850-A1-FP	5' -ATTTCGAAGCCTTATGATGGA-3'	800
UBC850-A1-RP	5' -TCACCTTGTTAATTAGATCGA-3'	

3.2.5. Validation of PCR markers for mating type identification

Mating type-specific primers PCAP1 (5'-ACGAGTACGAGTGCTTGGT-3') and PCAP2 (5'-TGAGTCTCGAGACAGAGAG-3') reported earlier by Li *et al.* (2017) were used to identify mating types of *P. capsici*/*P. tropicalis* isolates listed in Table 3.1. These are *P. capsici* specific primers which produce 508 bp band for A2 mating type and 997 bp band for A1 type. PCR was set up with a volume of 20 µL reaction mix composed of 2 µL of Taq Buffer A (Tris with 15mM $MgCl_2$, GeNei^{TM}), 0.5 µL of 2mM dNTP Mix (Thermo Scientific), 0.5 of 10 µM each of forward and reverse primers, Taq DNA Polymerase (1 U/ µL) and 1 µL of 100ng/µL of template DNA. PCR was carried out in Applied Biosystems^{TM} ProFlex^{TM} PCR System by following the temperature profile of initial denaturation at 94^{o}C for 5 min, followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 56^{o}C for 30 sec, extension at 72^{o}C for 1 min, and final extension for 10 min. The amplicons were visualized by agarose gel electrophoresis with 1 % of agarose in 1X TAE buffer at 90 Volts for 2 h, and were photographed using UV trans-illuminator (G: Box Chemi XR5, Syngene).

3.3. Results

3.3.1. Identification of *Phytophthora* at the species level

Phytophthora isolates infecting black pepper were identified at the species level using Ypt 1 gene-based species-specific primers viz., PC-Y-FP & COM-Y-RP2, PT-Y-FP & COM-Y-RP2 (Jeevalatha *et al.,* 2021), which amplify the DNA of *P. capsici* and *P. tropicalis* isolates respectively and ITS-based primers such as PP-F & COM-R, PN-F & COM-R, PMPCO-F & COM-R, which amplify the DNA of *P. palmivora, P. nicotianae* and *P. meadii* respectively. PC-Y-FP & COM-Y-RP2, PT-Y-FP & COM-Y-RP2 produced amplicon with 302 bp for *P. capsici* and 301 bp for *P. tropicalis* respectively and the rest of the primers produced the amplicon with 320 bp. The PCR amplicons produced by the species-specific primers were depicted in Fig. 3.3. It was identified that the isolates 98-48, 98-81, 98-87, 98-142, 98-155, 98-164, 98-174, 98-176, 98-185, 98-183, 98-198, 99-132, 00-42, 01-04, 02-20, 03-02, 03-10, 05-06, 05-09, 05-13, 05-14, 05-19, 06-01, 06-02, 06-03, 06-04, 06-09, 06-12, 06-13, 07-02, 07-05, 07-06, 07-07, 08-01, 08-02, 08-03, 08-05, 09-02, 09-03, 09-15, 10-02, 11-11, 11-13, 11-16, 11-17, 13-17, 13-21, 13-29, 13-30, 13-40, 13-41, 13-42, 13-46, 13-48, 18-01, 18-02, 18-09, 18-11, 18-12, 18-13, 18-20, 20-01, 20-02, 20-03, 20-04, 20-05, 20-06, 20-07, 21-01, 21-02, 22-01, 22-02, 22-03 and 23-01 were *P. capsici,* and the isolates 97-11, 97-19, 97-52, 97-53, 97-54, 97-55, 98-02, 98-03, 98-07, 98-60, 98-70, 98-76, 98-17, 98-59, 98-66, 98-65, 98-92, 98-93, 98-71, 98-128, 98-135, 98-143, 98-145, 98-156, 98-157, 98-171, 98-172, 98-175, 98-177, 98-182, 98-184, 99-91, 99-124, 99-136, 99-139, 99-144, 99-145, 99-166, 00-38, 01-20, 03-07, 05-17, 06-10, 06-11, 06-17, 08-07, 09-01, 09-08, 09-11, 09-13, 09-18, 09-19, 09-25, 09-26, 09-36, 09-39, 11-14, 11-19, 11-20, 11-21, 11-28, 11-29, 11-30, 11-32, 13-07, 13-09, 13-10, 13-11, 13-12, 13-23, 13-24, 13-33, 13-34, 13-36, 13-37, 13-45, 13-49, 13-51, 13-52 and 13-53 were *P. tropicalis.* The isolates 99-188, 09-03, 09-10, 09-22 and 09-27 were *P. meadii*. 98-95, 02-17 and 98-186 were *P. nicotianae* and 13-38 was *P. palmivora*. Out of a total of 164 isolates, 80 were *P. tropicalis*, 75 were *P. capsici*, 5 were *P. meadii,* 3 were *P. nicotianae* and 1 was *P. palmivora* (Fig. 3.3). The number and distribution of *Phytophthora* isolates collected across black pepper

growing regions in south India were given in Fig. 3.1. The species distribution based on the geographical area was illustrated using the sunburst chart (Fig. 3.4).

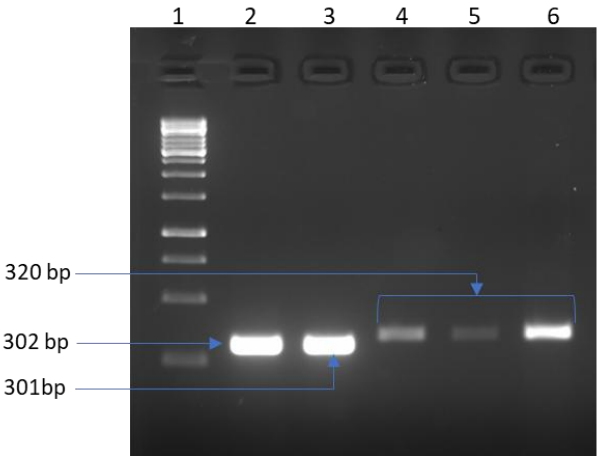


Fig. 3.2. Agarose gel showing the amplicons with species specific primers: Lane 1- 1 kb ladder, Lane 2- PC-Y-FP & COM-Y-RP2, Lane 3- PT-Y-FP & COM-Y-RP2, Lane 4- PN-FP & COM-R, Lane 5- PP-FP & COM-R, Lane 6- PMPCO & COM-R

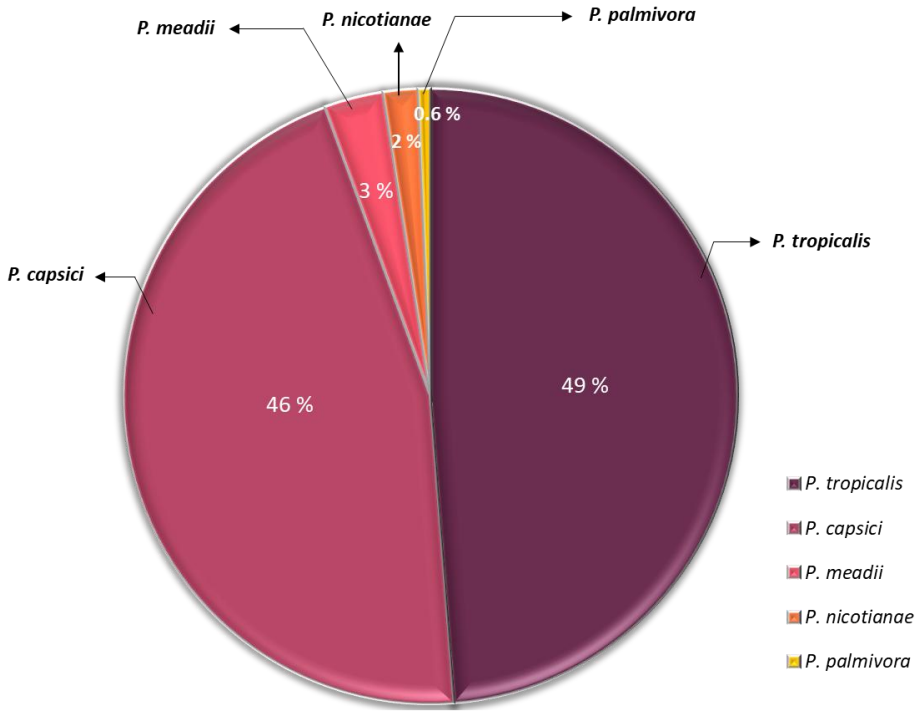


Fig. 3.3. Pie chart showing the abundance of *Phytophthora* species infecting black pepper

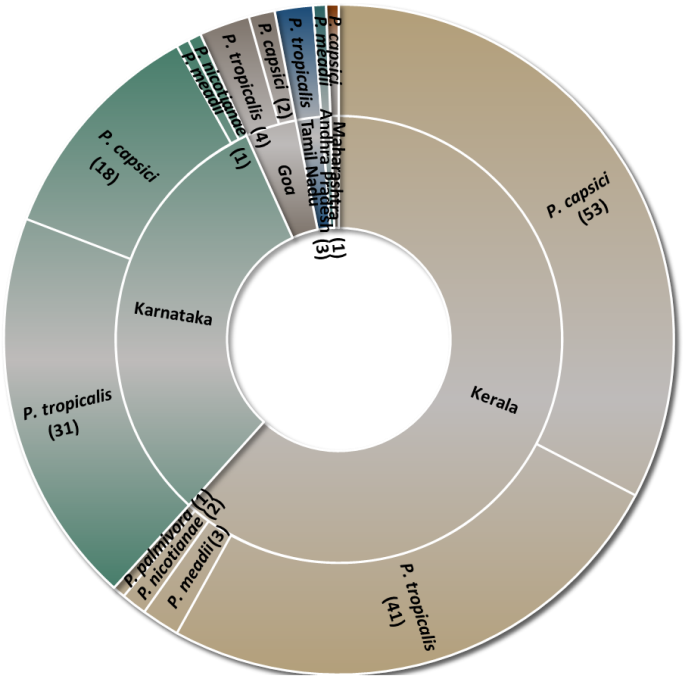
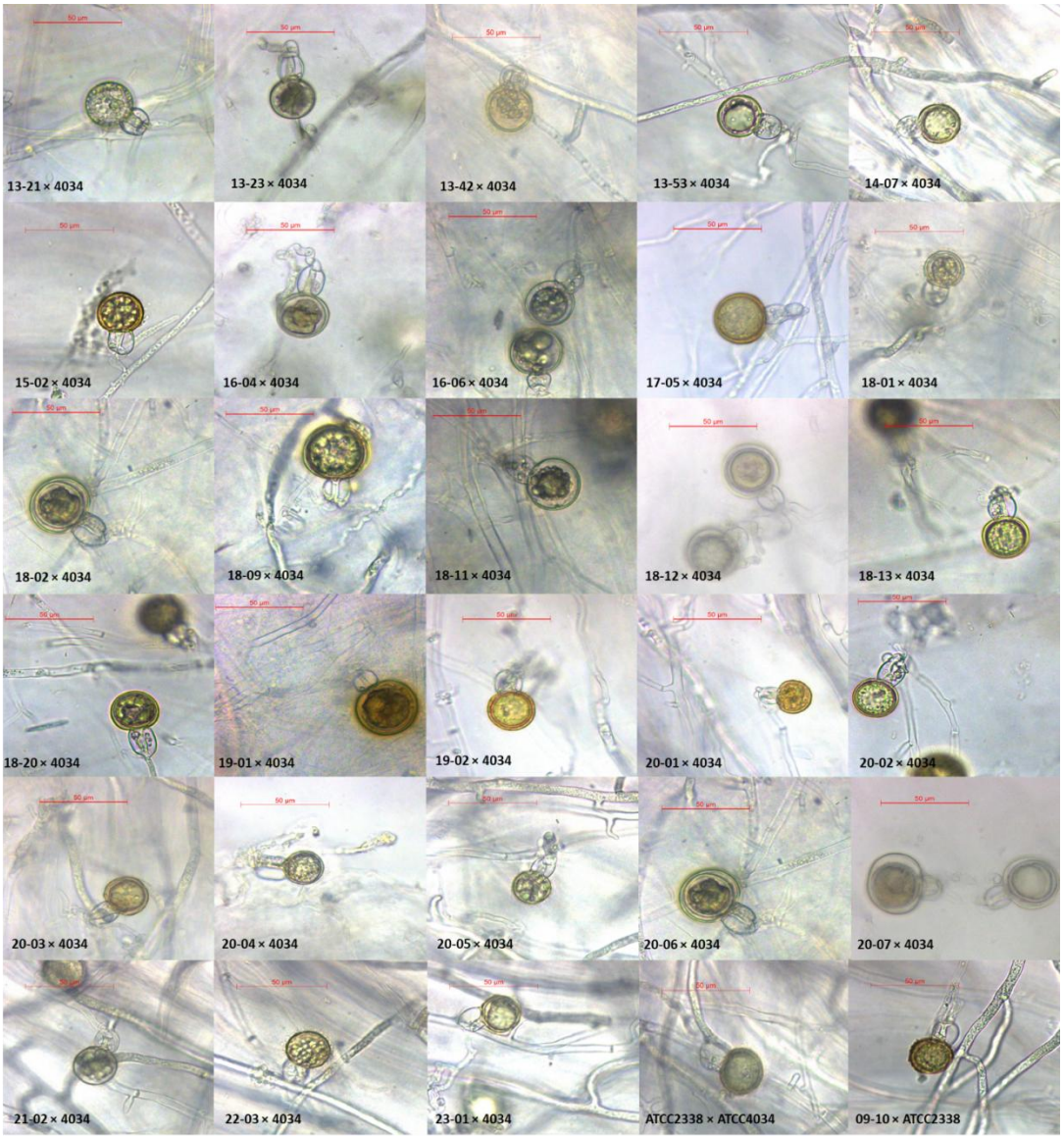


Fig. 3.4. Sunburst chart depicting the distribution of *Phytophthora* species in various locations: Kerala- *P. capsici* (53), *P. tropicalis* (41), *P. meadii* (3), *P. nicotianae* (2), *P. palmivora* (1); Karnataka- *P. capsici* (18), *P. tropicalis* (31), *P. meadii* (1), *P. nicotianae* (1); Goa- *P. capsici* (2), *P. tropicalis* (4); Tamil Nadu- *P. tropicalis* (3); Andhra Pradesh- *P. meadii* (1); Maharashtra- *P. capsici* (1)

3.3.2. Identification of mating type

The absence or presence of oospores from the pairing between the black pepper isolates with the reference isolates confirms the mating type of the isolates. The details of the pairing test observations performed in this study are given in Tables 3.6 & 3.7. Here, all the black pepper isolates produced oospores with A2 isolate (ATCC-MYA-4034) and not with A1 isolate (ATCC-MYA-2338), which shows that these isolates are of A1 mating type (Fig. 3.5).


13-21 × 4034
13-23 × 4034
13-42 × 4034
13-53 × 4034
14-07 × 4034
15-02 × 4034
16-04 × 4034
16-06 × 4034
17-05 × 4034
18-01 × 4034
18-02 × 4034
18-09 × 4034
18-11 × 4034
18-12 × 4034
18-13 × 4034
18-20 × 4034
19-01 × 4034
19-02 × 4034
20-01 × 4034
20-02 × 4034
20-03 × 4034
20-04 × 4034
20-05 × 4034
20-06 × 4034
20-07 × 4034
21-02 × 4034
22-03 × 4034
23-01 × 4034
ATCC2338 × ATCC4034
09-10 × ATCC2338

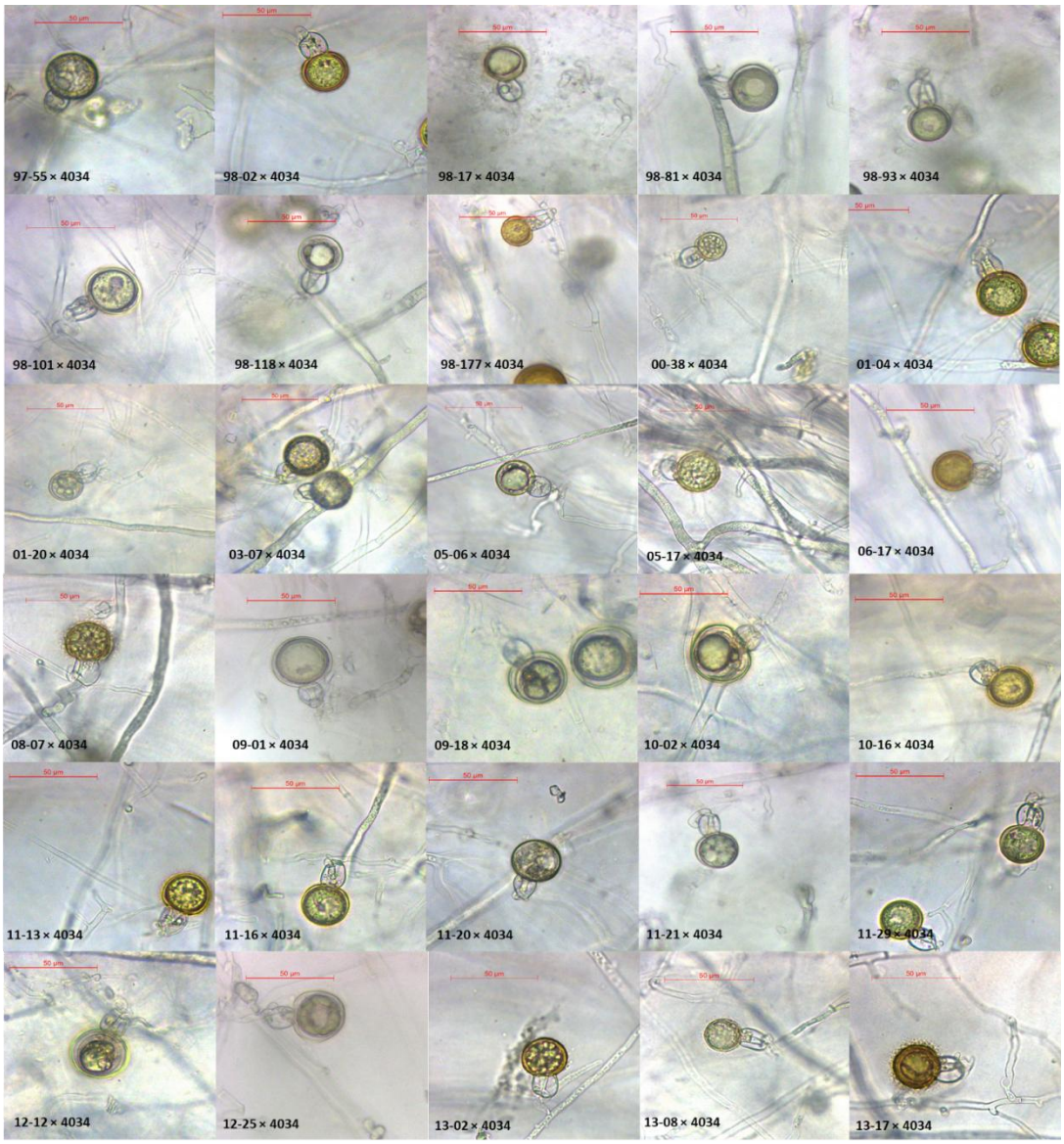


Fig. 3.5. Microscopic view of the oospores produced with the compatible mating types

Table 3.6. List of isolates used for pairing test and the production of oospores with the compatible isolates

Sl. No.	Isolate	Selfing	Mating with ATTC2338 (A1)	Mating with ATTC4034(A2)	Remarks
1	97-55	*absent	*absent	*present	A1
2	98-02	absent	absent	present	A1
3	98-17	absent	absent	present	A1
4	98-81	absent	absent	present	A1
5	98-93	absent	absent	present	A1
6	98-101	absent	absent	present	A1
7	98-118	absent	absent	present	A1
8	98-177	absent	absent	present	A1
9	00-38	absent	absent	present	A1
10	01-04	absent	absent	present	A1
11	01-20	absent	absent	present	A1
12	03-07	absent	absent	present	A1
13	05-06	absent	absent	present	A1
14	05-17	absent	absent	present	A1
15	06-17	absent	absent	present	A1
16	08-07	absent	absent	present	A1
17	09-01	absent	absent	present	A1
18	09-10	absent	present	absent	A2
19	09-18	absent	absent	present	A1
20	10-02	absent	absent	present	A1
21	10-16	absent	absent	present	A1
22	11-13	absent	absent	present	A1
23	11-16	absent	absent	present	A1
24	11-20	absent	absent	present	A1
25	11-21	absent	absent	present	A1
26	11-29	absent	absent	present	A1
27	12-12	absent	absent	present	A1
28	12-25	absent	absent	present	A1
29	13-02	absent	absent	present	A1
30	13-08	absent	absent	present	A1
31	13-17	absent	absent	present	A1
32	13-21	absent	absent	present	A1
33	13-23	absent	absent	present	A1
34	13-42	absent	absent	present	A1
35	13-53	absent	absent	present	A1

36	14-07	absent	absent	present	A1
37	15-02	absent	absent	present	A1
38	16-04	absent	absent	present	A1
39	16-06	absent	absent	present	A1
40	17-05	absent	absent	present	A1
41	18-01	absent	absent	present	A1
42	18-02	absent	absent	present	A1
43	18-09	absent	absent	present	A1
44	18-11	absent	absent	present	A1
45	18-12	absent	absent	present	A1
46	18-13	absent	absent	present	A1
47	18-20	absent	absent	present	A1
48	19-01	absent	absent	present	A1
49	19-02	absent	absent	present	A1
50	20-01	absent	absent	present	A1
51	20-02	absent	absent	present	A1
52	20-03	absent	absent	present	A1
53	20-04	absent	absent	present	A1
54	20-05	absent	absent	present	A1
55	20-06	absent	absent	present	A1
56	20-07	absent	absent	present	A1
57	21-02	absent	absent	present	A1
58	22-03	absent	absent	present	A1
59	23-01	absent	absent	present	A1
60	ATCC2338	absent	absent	present	A1
61	09-26 (brinjal)	absent	absent	absent	
62	19-173 (tomato)	absent	absent	absent	
63	99-191(tomato)	absent	absent	absent	

*absent/*present – absence or presence of oospore.

Table 3.7. List of isolates used for pairing test and the production of oospores with the compatible isolates

Sl. No.	Isolate	Pairing with other isolates	Presence of oospores	Remarks
1	98-93	ATCC76651 (A1)	absent	A1
2	97-55	ATCC76651 (A1)	absent	A1
3	11-29	ATCC76651 (A1)	absent	A1
4	98-02	ATCC76651 (A1)	absent	A1
5	98-177	ATCC76651 (A1)	absent	A1
6	11-21	ATCC76651 (A1)	absent	A1
7	13-23	ATCC76651 (A1)	absent	A1
8	13-53	ATCC76651 (A1)	absent	A1
9	13-09	ATCC76651 (A1)	absent	A1
10	13-07	ATCC76651 (A1)	absent	A1
11	13-33	ATCC76651 (A1)	absent	A1
12	05-06	ATCC52239	absent	A1
13	ATCC2338	ATCC52239	absent	A1
14	05-06	97-55 (A1)	absent	A1
15	05-06	98-177 (A1)	absent	A1
16	98-177	09-10 (A2)	present	A1
17	05-06	98-93	absent	A1

3.3.3. Development of mating type-specific marker

Isolates representing A1 and A2 mating types were used in PCR assay with ISSR primers. Unique bands were observed with primers viz. UBC850, UBC851, ISSR4, (TG)7 and (GA)8C which are shown in Fig. 3.6. Analysis of sequences of these unique bands from 05-06 and ATCC-MYA-4034 showed that they are from different regions, and therefore the primers designed based on these regions could not differentiate A1 and A2 mating types. Similar-sized bands were produced in both A1 and A2 mating type isolates (Fig. 3.7).

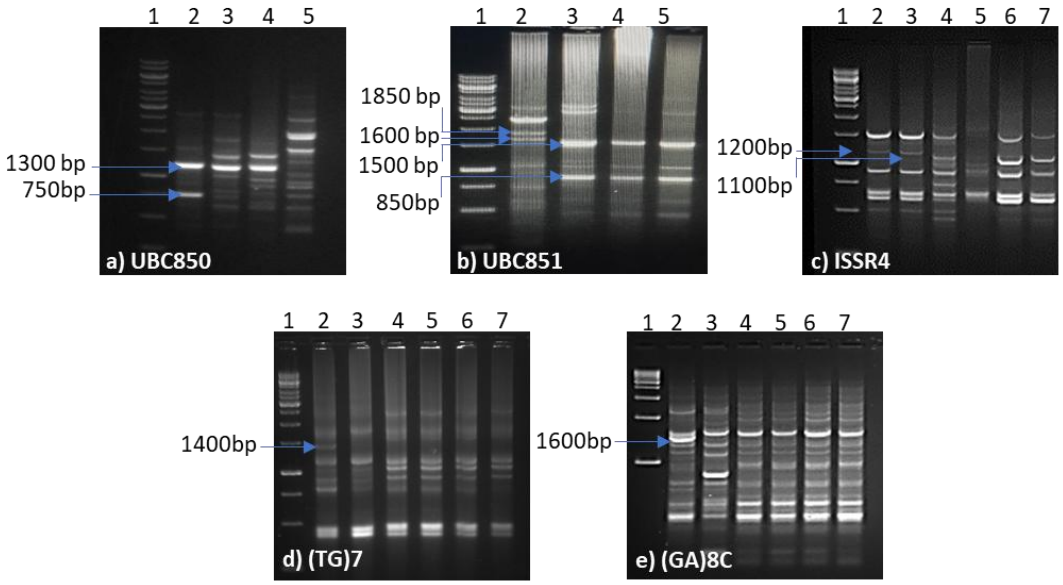


Fig. 3.6. Agarose gel image showing unique bands in A1 and A2 mating types, lane 1- 1kb ladder, lane 2- ATCC4034 (A2), lane 3- ATCC2338 (A1), lane 4- 05-06, lane 5- 20-05, lane 6- 18-12, lane 7- 22-01 a) UBC850; b) UBC851; c) ISSR4; d) (TG)7; e) (GA)8C

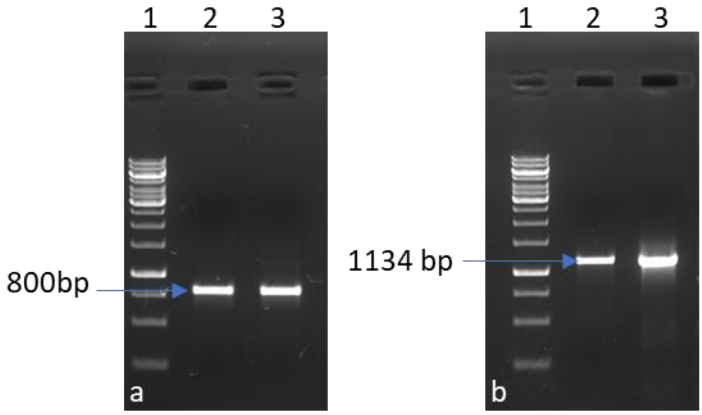


Fig. 3.7. Agarose gel showing the amplicons in A1 and A2 mating type with a) IS4-FP & IS4- RP; b) UBC850-A1-FP & UBC850-A1-RP. Lane 1- 1kb ladder, Lane 2- ATCC4034, Lane 3- 05-06

3.3.4. Validation of reported PCR markers for mating type identification

Among the 164 *Phytophthora* isolates analyzed, all 75 *P. capsici* isolates produced the products with a band size of 508 bp (Fig. 3.8a). Isolates which belonged to other species did not show any amplification with the PCAP1 and PCAP2 primers. PCR assay was also performed with reference isolates ATCC-MYA-4034, ATCC-MYA-

4043, ATCC-MYA-76651, ATCC-MYA-52239, ATCC-MYA-2338 (obtained from American Type Culture Collection, Manassas, USA. www.atcc.org) and it is observed that the reference isolates ATCC 4034 and ATCC 2338 which are of A2 and A1 mating type respectively produced amplicons of 997 bp (Fig. 3.8b) and other ATCC isolates did not amplify as they were the members of different species, i.e., ATCC 4043 is *P. meadii*, ATCC 52239 is *P. palmivora* and ATCC 76651 is *P. tropicalis.*

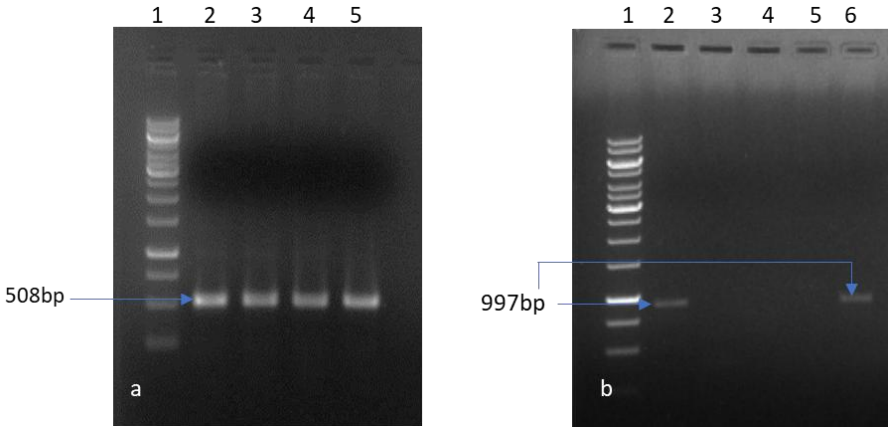


Fig. 3.8. Agarose gel image showing the amplicons with PCAP 1 and PCAP2 primers a) Black pepper *Phytophthora* isolates; b) Reference isolates ATCC cultures ATCC4034, ATCC4043, ATCC76651, ATCC52239, ATCC2338

3. 4. Discussion

Foot rot caused by *P. capsici* and *P. tropicalis* is one among the serious diseases of black pepper as it inflicts rampant setbacks in the field if left unnoticed. The accurate and timely identification of the pathogen is very crucial for the management of the disease. The present study aimed to study the different species associated with the foot rot disease of black pepper in India and its distribution and mating type identification. It was observed that in India, the species associated with foot rot disease were *P. capsici* and *P. tropicalis* and were comparably equal in number, though other species like *P. palmivora*, *P. meadii* and *P. nicotianae* were also identified in few numbers during the period from 1997 to 2024. There are reports which show the association of other species of *Phytophthora,* as well as foot rot disease other than the aforementioned ones, namely *P. parasitica* and *P.*

citrophthora (Anandaraj, 2012). Although the number of reports showing the association of *P. tropicalis* was not so much, it was observed that in the present study, the number of *P. tropicalis* identified was slightly greater than that of *P. capsici,* but of late the occurrence of *P. capsici* species is more.

As the morphological features of both *P. capsici* and *P. tropicalis* are similar, their distinction based on morphology is difficult (Orlikowski *et al.,* 2006). In this scenario, molecular techniques for species identification sounded to be more promising (Bhai *et al.,* 2022). Ypt 1 gene-based species-specific primers (Jeevalatha *et al.,* 2021) and ITS-based primers were used for the accurate distinction of species in the present study. The distribution pattern of the *Phytophthora* species across major black pepper growing states shows that a greater number of *P. capsici* were isolated from Kerala and *P. tropicalis* was isolated from Karnataka. The number of isolates obtained from Tamil Nadu was very less and all of them were *P. tropicalis*. The occurrence of other *Phytophthora* species was rare, i.e., among 9 isolates, 5 were *P. meadii,* of which 3 were from Kerala and one each from Karnataka and Andhra Pradesh. Only one *P. palmivora* isolate was collected, which is from Kerala. *P. nicotianae* was isolated from both Kerala as well as Karnataka.

The mating type of the black pepper isolates of *P. capsici* and *P. tropicalis* from India was identified as A1 in the present study. However, the presence of A2 mating type of *P. capsici* infecting cocoa from Kerala and A1 and A2 types of *P. palmivora* from Kerala and Karnataka was reported earlier by Chowdappa and Chandramohanan (2002). However, there were no reports on the prevalence of *P. tropicalis* mating type. Heterothallic *Phytophthora* species do not have a reproductive barrier and the presence of both mating types in the same area can pause a threat to the rise of new species via interspecific hybridization. Hybrids show a wide host range as it can infect the host of their parents as well as its own host range, except in a few cases where it fails to infect the parental host (Jafari *et al.,* 2020). Number of interspecific hybrids have been reported in Europe, for instance the cross between *P. cambivora*- like and *P. fragariae* of alder trees (Brasier *et al.,* 1999), P. *capsici* and *P. tropicalis* (Donahoo & Lamour, 2008), *P. infestans*

and *P. mirabilis* (Goodwin & Fry, 1994), *P. lacustris* and *P. riparia* (Bourret *et al.,* 2020), *P.* x *alni* from the cross between *P. uniformis* and *P*. x *multiformis* (Mizeriene *et al.,* 2020).

Several attempts have been made in designing mating type specific markers, A2 mating type specific primer using Amplified Fragment Length Polymorphism (AFLP) (Kim & Lee, 2002), A1 mating type specific primer was designed using Sequence Characterized Amplified Region (SCAR) marker (Zhang *et al.,* 2006), A1 and A2 mating type specific primer of *P. capsici* was designed by Li *et al* (2017) using Inter Simple Sequence Repeat (ISSR) markers. Brylińska *et al.* (2018) used W16, S1 and PHYB markers in *P. infestans,* based on the mating type-specific locus S1 (Judelson, 1996), S1a/S1b primers supplemented with PinfTQ R/F (Mabon *et al.,* 2021). The present study made an attempt to design the mating type-specific primer using ISSR markers and also validated the primers designed by Li *et al*. (2017). The unique bands distinguishing both A1 and A2 mating types was cloned, sequenced and primers were designed, but these primers failed to distinguish both the A1 and A2 mating types in *P. capsici* and *P. tropicalis*. The reason for this is that one of the isolates that we selected for A2 mating type i.e., ATCC-MYA-4034 was from *Capsicum annum*, since A2 mating type from *Piper nigrum* was not identified so far, and a significant interspecies difference in the banding pattern was also observed. Further, the validation of ISSR based primer designed by Li *et al*. (2017) showed that the black pepper isolates of *P. capsici* which were proved to be A1 showed an amplicon of 508 bp (which was in the range of A2 mating type) and the ATCC 4034 and ATCC 2338 which were originally A2 and A1 mating type respectively were amplified at 977bp (which was the expected amplicon size of A1 mating type). Hence, the primer designed for *P. capsici* infecting *Capsicum annum* was found to be unsuccessful or not suitable for mating type identification in black pepper *P. capsici* isolates.

3.5. Conclusion

Early detection and timely action against foot rot disease are crucial in its management. In the present study, the major species of *Phytophthora* associated

with the disease in India are *P. capsici* and *P. tropicalis* and other species, namely, *P. nicotianae, P. palmivora* and *P. meadii* were identified in smaller numbers. Both *P. capsici* and *P. tropicalis* isolates obtained were almost equal in numbers. In the initial years, a greater number of *P. tropicalis* isolates were observed, whereas of late *P. capsici* isolates were more. All the isolates were identified to be A1 mating type based on pairing test with reference cultures. An attempt to develop mating type-specific marker was unsuccessful, and validation of the existing primers showed that they were not reliable in detecting mating type in *P. capsici* isolates infecting black pepper.

CHAPTER 4

DETERMINATION OF GENOME SIZE OF *PHYTOPHTHORA* ISOLATES AND ITS CORRELATION WITH VIRULENCE AND FUNGICIDE RESISTANCE

Abstract

The prevalence of a pathogen in the field and its inter and intra-species relatedness has a prime role in its genetic composition. Identification of genome size of an organism helps in understanding the genetic variations associated with it, which were most often attributed to mutational changes in the genome like insertion, deletion and substitution and is vital in gaining clue about the ability of the pathogen to withstand adverse environmental conditions, fungicide resistance and variations in the pathogenicity exhibited by the pathogen. A deeper insight on genome size and its relationship with fungicide resistance will facilitate to develop effective management programmes. So, in the present study, we have analyzed the genome size of the *Phytophthora* isolates infecting black pepper using flow cytometry. Variations were observed in the genome sizes of the analyzed isolates, moreover, the genome size of *P. capsici* isolates was found to be slightly greater than *P. tropicalis* isolates. Further, some of its characters such as hyphal growth, release of zoospores and virulence analysis were performed using standard protocols. It was found that mycelial growth was greater for PC21-02 and PT98-93 and low for PT97-55 and PT03-07 whereas the zoospore release was greater for PC22-03 and low for PT97-55. Although the isolates PT97-55 and PT03-07 had comparatively slower mycelial growth and low zoospore release, PT97-55 were more virulent than PT03-07 to some extent. The fungicide sensitivity analysis of these isolates against the commonly recommended fungicides showed that all the isolates were sensitive to metalaxyl-mancozeb, copper oxychloride, Bordeaux mixture and less sensitive to potassium phosphonate at the recommended concentrations.

4.1. Introduction

Identification of genome size of an organism helps in understanding the genetic variations associated with it, which were most often attributed to mutational changes in the genome like insertion, deletion and substitution and is vital in gaining clue about the ability of the pathogen to withstand adverse environmental conditions, fungicide resistance and variations in the pathogenicity exhibited by the pathogen. The Genome size of an organism is the total DNA contained in the nucleus of haploid genome (C-value) (Elliott & Gregory, 2015) which is usually expressed as numbers in base pairs/kilobases/megabases or as mass in picograms (Sessions, 2013).

Various approaches were reported to study the genome size of organisms viz. read depth mapping (Pflug *et al.,* 2020), k-mer distribution (Next- generation sequencing), flow cytometry (Pflug *et al.,* 2020; Al-Qurainy *et al.,* 2021; He *et al.,* 2016), Real-time PCR (Wilhelm *et al.,* 2003), qPCR (Kadota *et al.,* 2023; Gregory *et al.,* 2013), Low coverage based Genome Size Estimator (LocoGSE) (Guenzi-Tiberi *et al.,* 2024), high contiguity and short read mapping (Natarajan *et al.,* 2019)

Flow cytometry is a popular technique used to analyze the physical and chemical aspects of a particle in suspension as it passes through the laser beam. Three components of flow cytometry include fluidics, optics and electronics, where the fluidics comprises a sheath fluid in which cells are suspended and carries the cells through the flow cell, where a single stream of cells produced by hydrodynamic focusing. The cells passing through the laser beam scatter the light and are collected by the Forward Scatter Channel (FSC), which describes the particle size, and the Side Scatter Channel (SSC) gives an idea about the granularity of the particle. When emitted light hits the detector, the voltage of the current generated was amplified and converted to electrical signals using Analogue to Digital Converters (ADC), and graphs were generated (Rahman, 2006).

Flow cytometry is a promising technique to study the ploidy, genome content (D'Hondt *et al.,* 2011), life cycle and colonization of a pathogen (Silva *et al.,* 2021) and hybridization in *Phytophthora* (Poucke *et al.,* 2021). It is the standard method

for genome content estimation as it rules out the errors that occur during sequencing (Van Poucke *et al.,* 2016). Smaller genome content can also be studied using flow cytometry (Čertnerová, 2022), instability and variation in single oospore progenies (Vercauteren *et al.,* 2011). Other applications of flow cytometry in pathology include testing and quantification of pathogens, viability studies and gene expression analysis using fluorescent probes (D'Hondt *et al.,* 2011)

Phytophthora is a pathogen with a dynamic genetic make-up. The population of *Phytophthora* infecting black pepper in India is genetically unique as identified from RAMS and REP-PCR analyses with a prominent inter and intra-species variation (Zumaila *et al.,* 2024). Frequent monitoring of the pathogen population is highly recommended to counter the emergence of fungicide resistant *Phytophthora* isolates. The present study focused on the analysis of genome size of *Phytophthora* spp. infecting black pepper using flow cytometry, and comparison of variation in its growth pattern, pathogenicity, fungicide sensitivity with the estimated genome size of the pathogen.

4.2. Materials and methods

4.2.1. Source and selection of isolates

The *Phytophthora* isolates from the year 1997 to 2023 used in the present study were obtained from the National Repository of *Phytophthora,* ICAR-IISR, Kozhikode (Table 4.1). The isolates which were selected after the year 2018 were retrieved from the infected tissue/rhizosphere soil of black pepper. Species identity for all the isolates was established through PCR using Ypt gene-based species-specific primers (Jeevalatha *et al.,* 2021) and ITS primers (Bhai *et al.,* 2022). Isolates were maintained in vials containing sterile distilled water and stored at 25±1^{o}C for long-term storage.

Table 4.1. *P. capsici* and *P. tropicalis* isolates used for the genome size estimation.

Sl. No.	Species	Name of isolate	Place of collection
1	*P. capsici*	01-04	Adivaram, Kozhikode
2	*P. capsici*	05-06	Peruvannamuzhi, Kozhikode
3	*P. capsici*	18-02	Plot 1, Kozhikode
4	*P. capsici*	18-12	Idukki
5	*P. capsici*	20-05	Panniyur, Kannur
6	*P. capsici*	21-02	Panniyur, Kannur
7	*P. capsici*	22-03	Maharashtra
8	*P. capsici*	23-01	Peruvannamuzhi, Kozhikode
9	*P. tropicalis*	97-55	Valparai, Coimbatore
10	*P. tropicalis*	98-93	Adyanadka, Dakshin Kannada
11	*P. tropicalis*	03-07	Adivaram, Kozhikode
12	*P. tropicalis*	06-17	Yercaud, Salem
13	*P. tropicalis*	09-01	Peruvannamuzhi, Kozhikode
14	*P. tropicalis*	11-29	Wayanad
15	*P. tropicalis*	13-23	Nedumkandam, Idukki
16	*P. tropicalis*	13-53	Goa

4.2.2. Preparation of isolates for the genome size analysis

The isolates listed in Table 4.1 were revived from the stored vials containing sterile distilled water by transferring the mycelial disc carefully onto a sterile blotting paper to remove the water content. The mycelial discs were then placed onto the Petri plate containing the carrot agar media and incubated at 25±1^{o}C. Discs of 5 mm were then cut out from the mycelial tip using a sterile cork-borer from the three-day-old cultures and were transferred to Ribeiro's broth for the increased mycelial growth, and growth conditions followed were same as above. All the above activities were performed inside the Igene Labserve Laminar Air flow chamber to prevent the chances of contamination.

4.2.3. Genome size estimation using flow cytometer

The genome size estimation was carried out at flow cytometry Solutions Pvt. Ltd, Rajasthan, India. As the genome size of these black pepper *Phytophthora* isolates had not been studied earlier, initially *Raphanus sativus* cv. Saxa (2C- 1.11 pg= 1086

Mb; Doležel *et al.,* 1992) was used as the internal standard, the outcome showed that the 2C content of *Phytophthora* is much less than that of *Raphanus sativus* cv. Saxa and hence it could not be used for the accurate estimation of the genome size as the internal standard was way too large than the unknown. Then, we used *Arabidopsis thaliana* ecotype Columbia (Col-0) with 2C= 0.322 pg as the internal standard for further analysis. The tender leaves of *Arabidopsis thaliana* were chopped finely along with the mycelium using a fine pair of scissors in 1 ml of nuclei isolation buffer. The suspension was filtered using a 10 µm cell strainer and collected into a 5 mL FACS tube. The suspension was incubated for 15 minutes, and the suspension with isolated nuclei was run on the flow cytometer. The reference control was used as an internal standard for the genome size estimation.

4.2.4. Data analysis

Data acquisition of the mixture of sample and reference control was obtained using flow cytometer equipped with a 552 nm laser and a 585/42 band pass filter. About 2000 nuclei were analyzed. The genome size of the internal standard (0.322 pg) was set on the detector, which was used to run the unknown sample. The results were generated as one-parameter histograms with G_0/G_1 on a linear scale. The fluorescent intensity (median) of G_0/G_1 peak of the reference control and the standard was examined. The genome size of the unknown samples was estimated using the formula:

Genome size of the sample = Genome size of the reference × (Median value of G_0/G_1 peak of the sample/ Median value of G_0/G_1 peak of the reference)

$$1 \text{ pg DNA} = 0.978 \times 10^9 \text{ bp}$$ (Doležel *et al.,* 2003)

4.2.5. Virulence analysis

To study the correlation between genome size and virulence, Sreekara and IISR Shakthi varieties of black pepper were used for the in vitro analysis. The actively growing mycelial disc of 5mm was cut out from the three-day-old culture. The second leaf of the plant was inoculated with the mycelial disc of the isolates and was covered with wet cotton to prevent the discs from drying. The leaves were then

placed inside an enclosed box sprayed with sterile distilled water to maintain humidity. The leaves inoculated with discs of carrot agar were served as control, and the tests were performed in triplicate. The plants were then observed for the visible symptoms on the subsequent days, and the lesion dimensions were recorded and ANOVA was carried out using R studio R version 4.2.1 (R Core Team 2022).

4.2.6. Hyphal growth and zoospore production

To measure the rate of hyphal growth, the 5 mm mycelial discs of *Phytophthora* isolates were placed on carrot agar medium and incubated at 25±1 °C. The hyphal growth was measured at 24 h intervals for three consecutive days, and the average of the three readings at each time interval was calculated for further analysis. Mycelial Growth Speed Index (Oliveira *et al.,* 2016) was calculated using the equation:

$$MGSI=[(D^{24h}-0) + (D^{48h}-D^{24h}) + (D^{72h}-D^{48h})]/3$$

Where D^{24h} is the average diameter after 24h of incubation, D^{48h} is the average diameter after 48h of incubation, and D^{72h} is the average diameter after 72h of incubation. ANOVA was performed using R studio R version 4.2.1 (R Core Team 2022).

4.2.7. Enumeration of zoospores

Sporulation of the isolates was induced by placing the 5 mm mycelial discs of three-day-old cultures in a Petri plate containing sterile distilled water. The plates were then placed under LED light for continuous illumination up to 72h. The discs were then checked for sporulation under Nikon Alphaphot-2 YS2 Microscope. The zoospore enumeration was performed according to Du *et al.* (2014) with slight modifications. Five of the sporulated discs were placed in a sterile test tube containing 1 mL of sterile distilled water and incubated at 4^{o}C for 30 min followed by incubation at room temperature for 30 min for the release of zoospores (Fig. 4.1). The zoospore suspension was stained with 0.1% of aniline blue and counted using a hemocytometer.

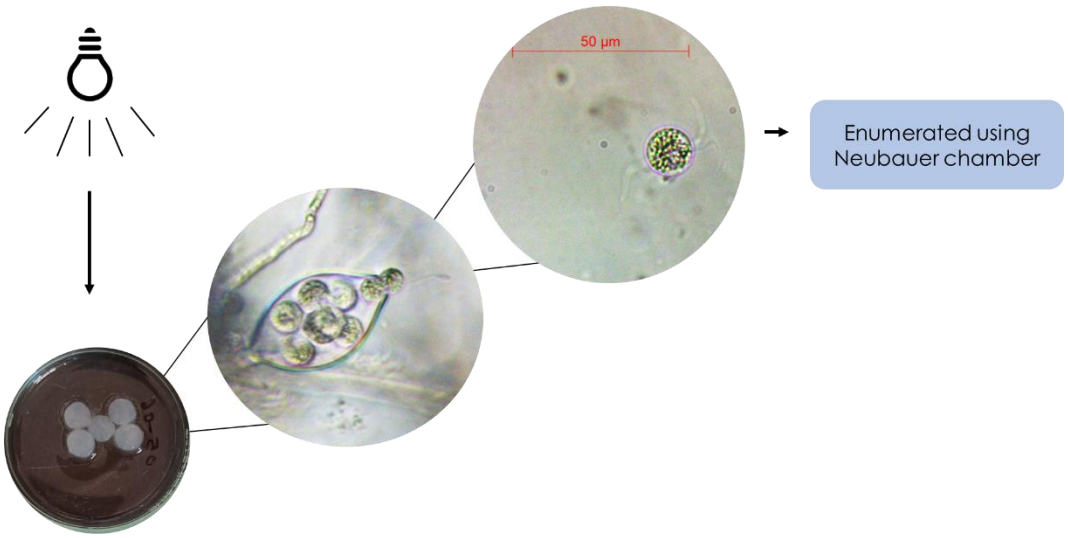


Fig. 4.1. Sporulation induction in mycelial discs of *Phytophthora,* sporangia showing zoospores release and zoospore at 40x magnification under a compound microscope.

4.2.8. Correlation with fungicide sensitivity

Fungicide sensitivity of the isolates was studied by the poison food technique (Sindhu *et al.,* 2022). Here, the carrot agar medium used for culturing the *Phytophthora* isolates was amended with the commonly used fungicides at their recommended concentrations (Table 4.2). The tests were carried out in triplicate, and the carrot agar (without fungicides) inoculated with the isolates served as a control. The readings were recorded when the mycelial growth covered the control plate. Per cent inhibition was calculated, and the data were subjected to ANOVA using R studio R version 4.2.1 (R Core Team 2022).

Table 4.2. List of commonly used fungicides and its recommended concentrations.

Sl. No.	Fungicide	Recommended concentration
1	Metalaxyl mancozeb	0.125%
2	Potassium phosphonate	0.3%
3	Copper oxychloride	0.25%
4	Bordeaux mixture	1%

4.2.9. Correlation analysis

The association among each of the variables, such as genome size, mycelial growth, zoospore release, virulence and fungicide sensitivity, were examined using

correlation analysis, which was performed by R studio R version 4.2.1 (R Core Team 2022).

4.3. Results

4.3.1. Selection and maintenance of isolates

The isolates which were obtained from the National Repository of *Phytophthora* ICAR-IISR and for those cultures which were isolated from the infected black pepper samples (Table 4.1), species identity was determined using the species-specific primers and were chosen for the genome size estimation analysis.

4.3.2. Sample preparation for flow cytometry analysis

All the selected isolates were revived in carrot agar media and were grown in Ribeiro's broth for the excess of mycelial production after three days. The mycelia were allowed to grow for about one week to get enough mycelial mass before performing the analysis.

4.3.3. Flow cytometry for genome size estimation

For the genome size analysis of *Phytophthora* isolates, initially *Raphanus sativus* cv. Saxa was used as an internal standard. However, due to its huge variation in the range of size, *Arabidopsis thaliana* ecotype Columbia (Col-0) with 2C = 0.322 pg was chosen as its genome size is within the range of *Phytophthora* genome size. The histogram of the G_0/G_1 peak of *Arabidopsis thaliana* ecotype Columbia (Col-0), when run alone is shown in Fig. 4.2. The median value was 39399.50 with the coefficient of variation not exceeding 5%. For the genome size estimation, the reference control *Arabidopsis thaliana* ecotype Columbia (Col-0) was run as an internal standard, i.e., the control was run together with the sample and the observations were recorded. The median value of the G_0/G_1 peak of the simultaneous run of control and the sample was obtained from the histogram (Fig. 4.3), and the genome size was calculated using the equation given above (Table 4.3). The genome size of the selected isolates was plotted in Figure 4.4.

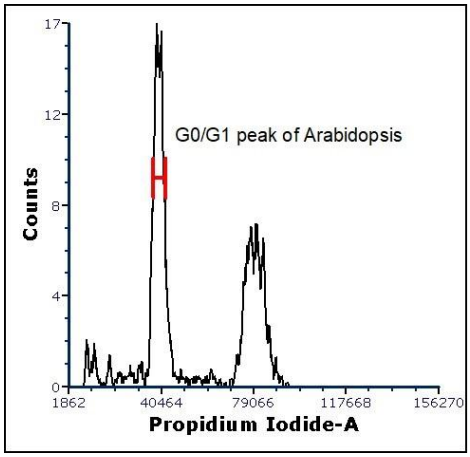


Fig. 4.2. Histogram showing the G_0/G_1 peak of *Arabidopsis thaliana* ecotype Columbia (Col-0) with 2C= 0.322 pg.

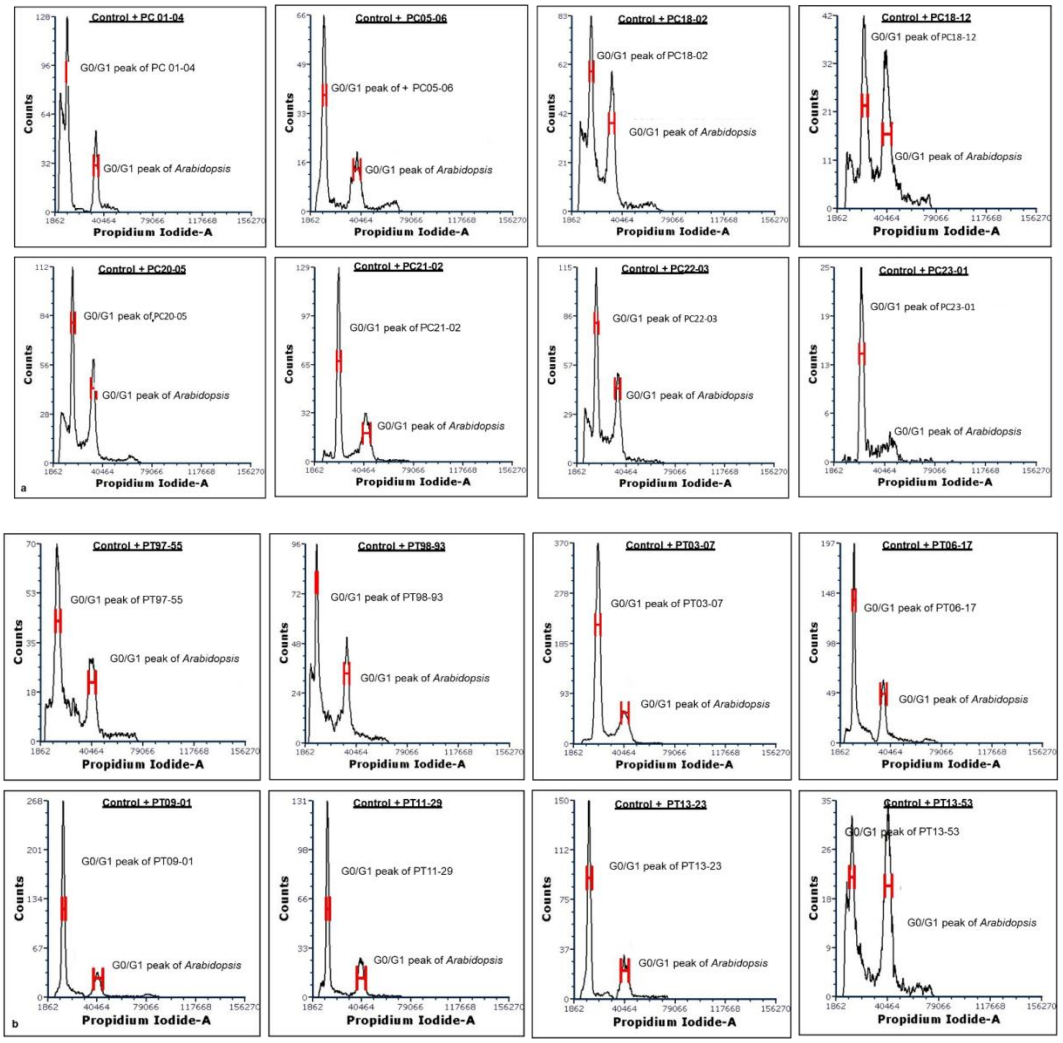


Fig. 4.3. Histogram showing the G_0/G_1 peak of control and the selected isolates: a) *P. capsici*; b) *P. tropicalis.*

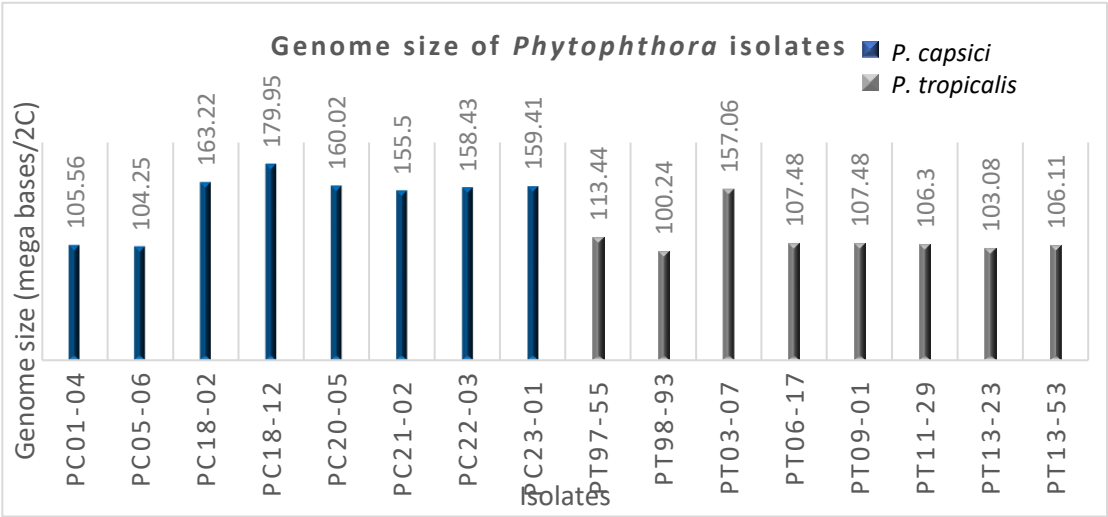


Fig. 4.4. Bar diagram showing the genome size of *P. capsici* and *P. tropicalis* isolates.

Table 4.3. Estimated genome size of the selected isolates.

Isolate	Median of G_0/G_1 of sample	Median of G_0/G_1 of standard	Genome size (pg)	Genome size (Mb)
PC01-04	11365.00	33894.00	0.108	105.62
PC05-06	12421.00	37493.00	0.106	104.25
PC18-02	16704.00	32221.00	0.167	163.33
PC18-12	22858.00	40009.00	0.184	179.95
PC20-05	16807.00	33015.00	0.164	160.39
PC21-02	20731.00	41949.00	0.159	155.50
PC22-03	17007.00	33825.00	0.162	158.43
PC23-01	20970.00	41457.50	0.163	159.41
PT97-55	14577.00	40495.00	0.116	113.44
PT98-93	10567.00	33183.00	0.102	099.76
PT03-07	20449.00	40995.00	0.160	157.06
PT06-17	12199.00	34645.00	0.110	107.58
PT09-01	13854.00	40562.00	0.109	107.48
PT11-29	13384.00	39645.00	0.108	106.30
PT13-23	13201.00	40327.50	0.105	103.08
PT13-53	13717.50	40711.50	0.108	106.11

Genome size of the isolates was analyzed by flow cytometry using *Arabidopsis thaliana* ecotype Columbia (Col-0) with 2C=0.322. The genome size of most of the *P. capsici* isolates were estimated to be greater than 150 Mbp/2C (155-180 Mbp) except for two isolates; PC01-04 and PC05-06 which showed around 105 Mbp (Table 4.3, Fig. 4.4). Whereas, in case of *P. tropicalis* the genome size was in the range of 103-107 Mbp/2C except for one isolate; PT03-07 (Table 4.3, Fig. 4.4).

4.3.4. Virulence analysis

Virulence analysis was performed by detached leaf assay using the black pepper varieties, Sreekara and IISR Shakthi (Fig. 4.5). The lesion diameters after 48 hours of inoculation was recorded and the lesion area was calculated using standard formula and statistically analyzed using ANOVA (Fig. 4.6). Among the *P. capsici* isolates, PC01-04 (18.48 cm^2) and PC20-05 (17.95 cm^2) were most virulent and PC05-06 (1.39 cm^2), PC18-12 (1.63 cm^2), PC18-02 (0.28 cm^2) and PC21-02 (0.12 cm^2) were less virulent in IISR Shakthi, whereas *P. capsici* isolates showed comparable pattern of virulence except for the isolates, PC18-02 (0.48 cm^2) and PC21-02 (0.24 cm^2) in Sreekara. In both varieties, *P. capsici* isolates, PC21-02 and PC18-02 showed minimum lesion area. In case of *P. tropicalis* isolates, PT06-17 (17.53 cm^2) and PT11-29 (13.81 cm^2) were most virulent, and PT03-07 (0.68 cm^2) and PT13-23 (0.46 cm^2) were least virulent in IISR Shakthi. Whereas, in case of Sreekara, PT97-55 (10.54 cm^2) and PT13-53 (7.46 cm^2) were more virulent, and PT03-07 (3.37 cm^2) and PT98-93 (2.74 cm^2) were least virulent.

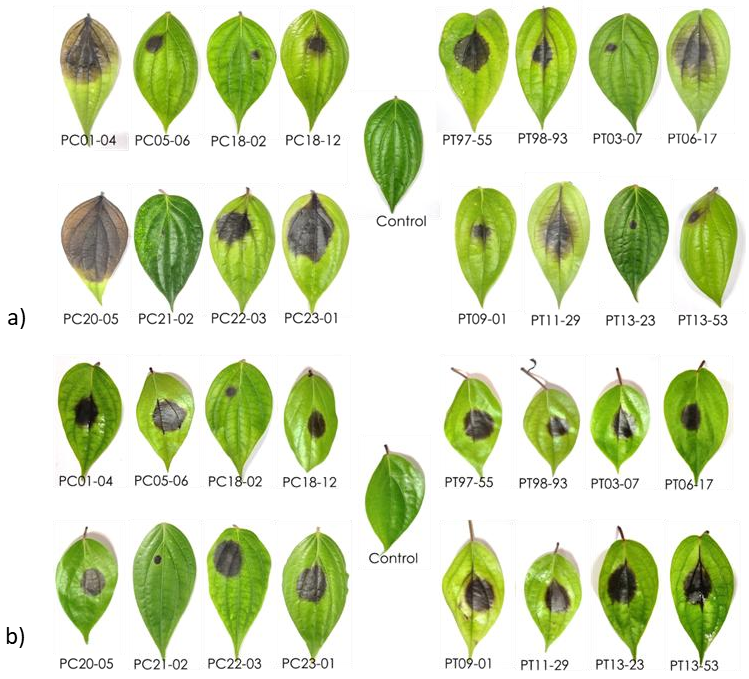


Fig. 4.5. Black pepper leaves showing the lesion developed upon inoculation of *Phytophthora* isolates (48h after inoculation): a) IISR Shakthi; b) Sreekara.

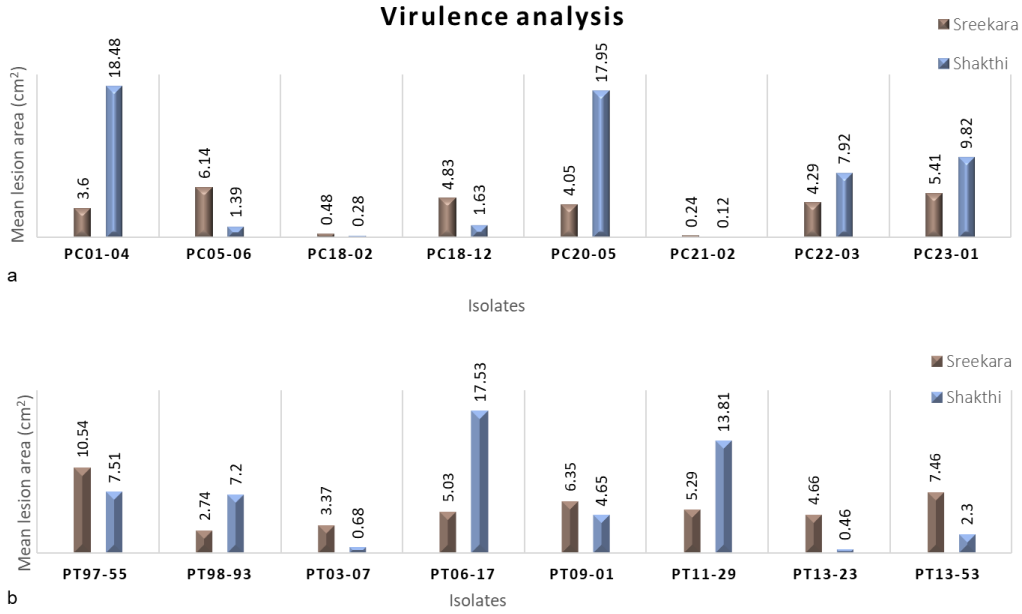


Fig. 4.6. Bar diagram showing the lesion area formed by *P. capsici* and *P. tropicalis* isolates at 48 h after inoculation.

4.3.5. Measurement of Hyphal growth

The MGSI ANOVA results were plotted in Fig. 4.7. The results showed that the mycelial growth speed was rapid for PC21-02 in the case of *P. capsici* isolates and in *P. tropicalis,* it was greater for the isolate PT98-93. The mycelial growth was slow for the isolates PC18-12 and PC18-02 in case of *P. capsici,* and in *P. tropicalis* it was for the isolates PT97-55, PT03-07 and PT11-29.

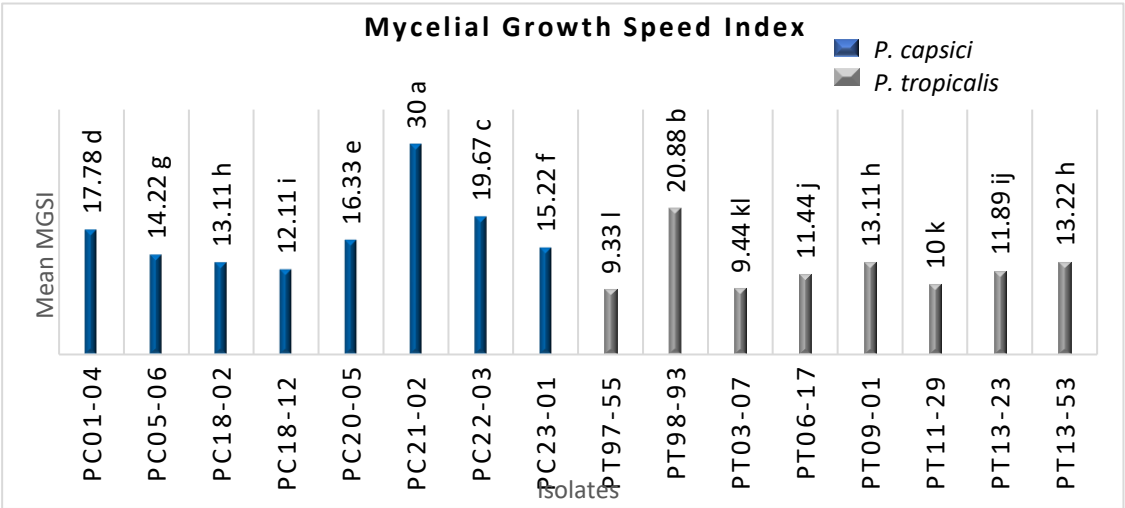


Fig. 4.7. Bar diagram showing the MGSI of *P. capsici* and *P. tropicalis* isolates.

4.3.6. Enumeration of released zoospores

Zoospores were counted by taking 10 µL of the zoospore suspension on a hemocytometer. Spores /mL were calculated using the formula given below and were analyzed using ANOVA (Fig. 4.8).

$$\text{Spores/mL} = \frac{\text{Total spores present in each of the quadrants}}{4} \times 10^4$$

It was observed that among *P. capsici*, the isolates PC22-03, PC20-05, PC18-12, PC01-04 and PC05-06 had comparatively greater release of zoospores. Whereas in *P. tropicalis*, it was greater for the isolates PT98-93, PT09-01, PT13-53 and PT16-17 and least for the isolates PT97-55, PT11-29 and PT03-07.

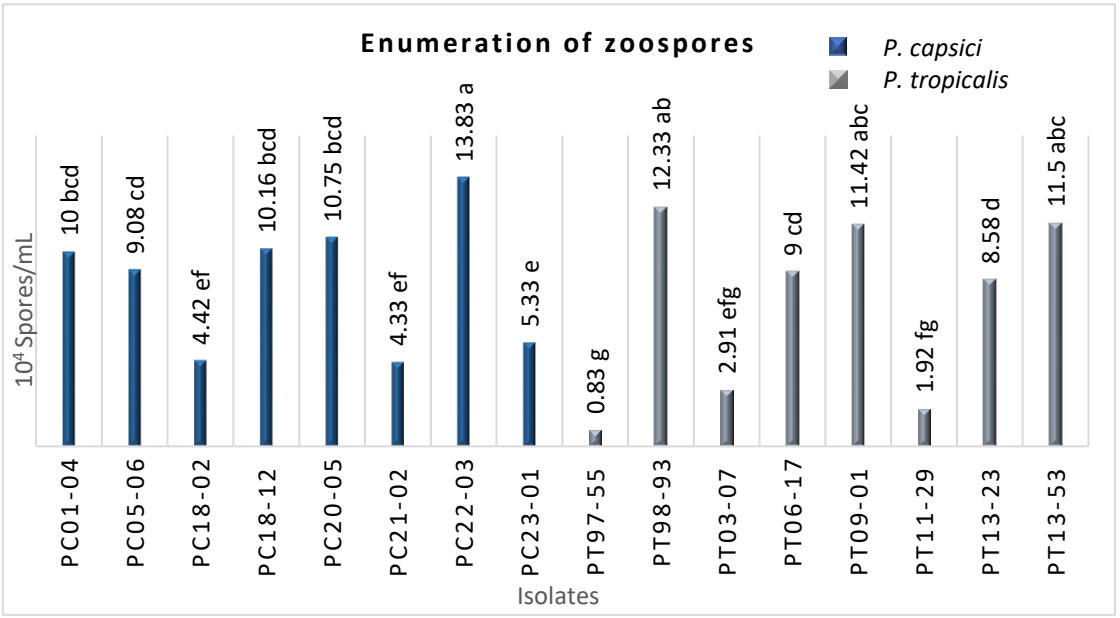


Fig. 4.8. Bar diagram showing the quantity of released zoospores of *Phytophthora* isolates.

4.3.7. Fungicide sensitivity

The test showed that all the fungicides except potassium phosphonate inhibited the growth completely. The percentage inhibition of potassium phosphonate was calculated, and ANOVA was used to examine the data, which were represented in Figure 4.9. It was also observed that potassium phosphonate showed complete inhibition in the case of PC18-12 and PT97-55.

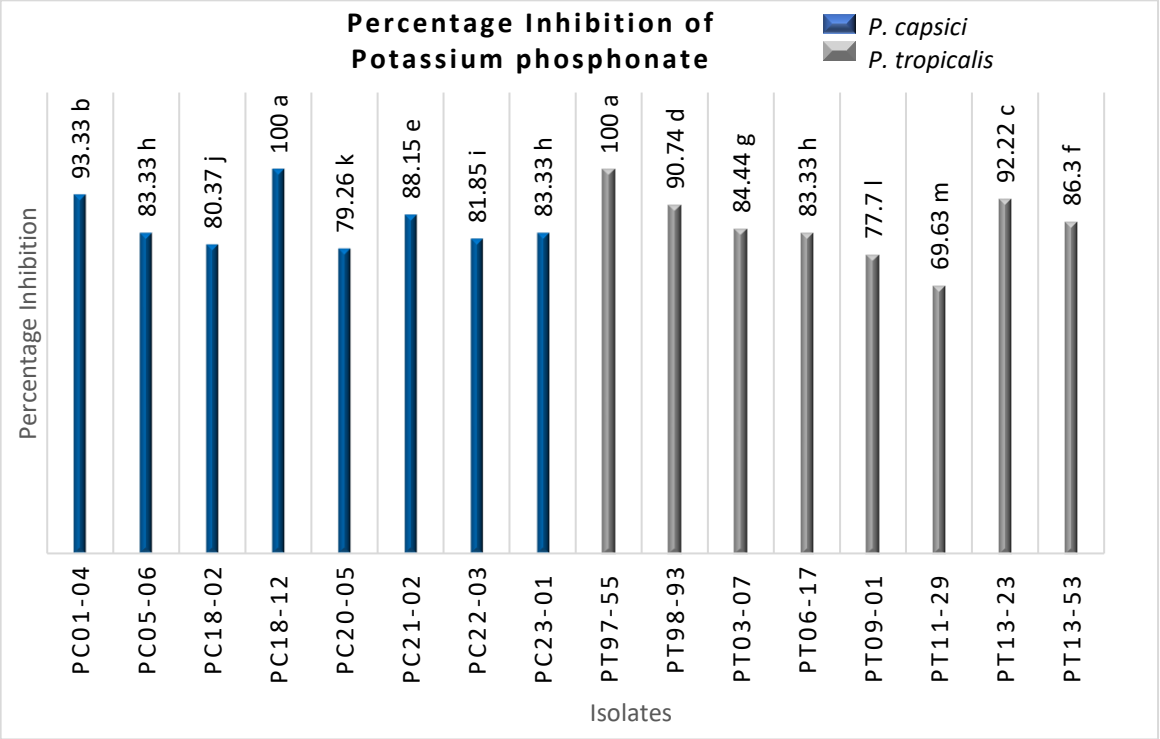


Fig. 4.9. Bar diagram showing the percentage inhibition of potassium phosphonate against *Phytophthora* isolates.

4.3.8. Correlation analysis

Correlation coefficient and its significance were computed and a correlogram was plotted in Figure 4.10. Where it can be seen that there exists no significant positive correlation among the variables, however, a weak positive correlation exists between mycelial growth and zoospore production and genome size and mycelial growth and a negative correlation can be observed between genome size and virulence and virulence and mycelial growth.

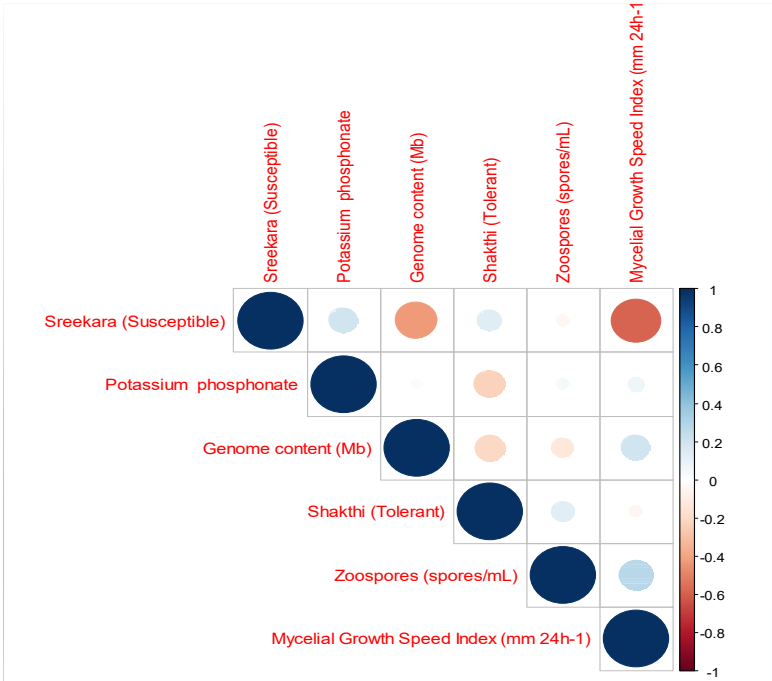


Fig. 4.10. Correlogram showing the correlation between the variables.

4.4. Discussion

Flow cytometry is one of the most widely accepted techniques when it comes to genome size/ploidy estimation, apart from the molecular techniques. There were several reports that studied the genome size of *Phytophthora* spp. using flow cytometry (Silva *et al.,* 2021; Wang *et al.,* 2016). Cui *et al.,* in 2019, used flow cytometry to confirm the results obtained by Oxford Nanopore Technology, where the genome size was found to be slightly lower than that obtained by flow cytometry. A similar trend was observed by Pflug *et al* (2020) in beetles. The results of the present study was almost identical to the reports mentioned above, where the whole genome size of *P. capsici* isolate PC05-06 was obtained as 80 Mbp and that of *P. tropicalis* was 74 Mbp (Unpublished data) obtained by next-generation sequencing and third-generation sequencing which were slightly lower than those observed from flow cytometry probably due to the overlaps generated in the long sequence reads or due to the erroneous system output caused by the signal generated by the corresponding nucleotide in case of repeat regions (Ari *et al.,* 2016; Cui *et al.,* 2019).

Two varieties of black pepper were used to study the virulence of the selected isolates using the detached leaf assay, and surprisingly, the results showed that those isolates which were more virulent in Sreekara (susceptible variety) were not that virulent in IISR Shakthi (tolerant variety) and vice versa. *In planta* studies showed that IISR Shakthi was tolerant in the preliminary screening (Bhai *et al.,* 2010). In case of susceptible variety, *P. tropicalis* isolate PT97-55 from Coimbatore district of Tamil Nadu was found to be most virulent. The least virulent isolates were PC18-02 and PC21-02 from Kozhikode and Kannur districts, respectively of Kerala. Whereas in tolerant variety, *P. capsici* isolate PC01-04 and PC20-05 of Kozhikode and Kannur districts of Kerala were most virulent and PC21-02 and 18-02 of Kannur and Kozhikode districts, respectively were least virulent. Among *P. tropicalis* isolates, PT06-17 from Salem district of Tamil Nadu and PT11-29 from Wayanad district of Kerala were more virulent, and PT03-07 and PT13-23 from Kozhikode and Idukki districts of Kerala were least virulent.

An interesting result was observed in Mycelial Growth Speed Index among the isolates, that it was greater for the isolate PC21-02 and least for PT97-55; however, the former was least virulent, whereas the latter was highly virulent. Yet another quantitative measurement of aggressiveness, i.e., in vitro zoospore release was greater for the isolates PC22-03, PT98-93, PT13-53 and PT09-01, although the virulence observed was moderate and was least for PT97-55 and PT11-29. The fungicides like copper oxychloride, metalaxyl-mancozeb, and Bordeaux mixture were highly efficient for its management and Potassium phosphonate however, it does not have a direct effect on *Phytophthora* could inhibit its growth to an extent.

The present study also attempted to identify the correlation between each of the variables analyzed. There was a significant negative correlation between virulence and one of the quantitative traits of aggressiveness i.e., mycelial growth speed. Tradeoffs were reported between aggressiveness and virulence; however, these were temporary (Fontyn *et al.,* 2024). Pathogen fitness and virulence both contribute towards the aggressiveness of a pathogen, and if the pathogen is aggressive, it need not be the virulent one (Pariaud *et al.,* 2009). Similar observations in *Plasmopara halstedii* were obtained by Sakr in 2011. The rest of the in vitro analyses were found to be unrelated towards each other.

4.5. Conclusion

The idea about the genome size of an organism would reinforce the knowledge on genetic diversity that it holds within. It is evident from the present study that the *Phytophthora* species infecting black pepper in India were diverse, and the observations were in compliance with the earlier reports, as the genome size among the isolates was not identical. Nevertheless, there is a clear distinction between the range of the genome size of two different species that have been discussed in the study. Moreover, the correlation analysis with virulence, aggressiveness and fungicide sensitivity indicated that there is no positive significant correlation among the variables; on the other hand, a negative correlation was spotted between the speed of mycelial growth and virulence. Other variables were unrelated. Also, the isolates did not acquire resistance towards the commonly recommended fungicides yet.

CHAPTER 5

GENETIC DIVERSITY AND PATHOGENICITY OF TWO *PHYTOPHTHORA* SPECIES INFECTING BLACK PEPPER IN INDIA

Abstract

The two major *Phytophthora* species associated with foot rot disease of black pepper in India are *Phytophthora capsici and P. tropicalis,* which pose a huge threat to its cultivation. There exists a significant genetic diversity amongst the pathogen, which is a crucial cause for its greater variability in the virulence pattern and its wide host range. Here, the genetic diversity of *Phytophthora* species was resolved using RAMS (Random Amplified Microsatellites) and REP (Repetitive Extragenic Palindromic)-PCR fingerprinting. *Phytophthora* isolates collected from major black pepper growing states such as Karnataka, Kerala, Tamil Nadu and Goa were used, out of which a total of forty-eight isolates, including 24 each of *P. capsici* and *P. tropicalis* isolates, were selected for the present study. The results disclosed a total of 160 loci, of which 150 loci (93.75%) were polymorphic. The data generated using UPGMA and PCoA, as stated by combined RAMS and REP-PCR data distinctly grouped the two species into two separate clusters, and further divided into 4 sub-clusters, namely I and II (*P. capsici*) and III and IV (*P. tropicalis*). The study also showed that the population was heterogeneous, with all the isolates being genetically unique. A greater number of polymorphic loci were observed for REP-PCR primers than that of RAMS primers. Moreover, morphological and infectivity analyses of the selected sixteen isolates were performed in vitro. All the isolates belonged to A1 mating type and displayed varied sporangial shapes and colony morphology. Infectivity analysis showed that the *Phytophthora* isolates produced symptoms in tomato, cucumber, pumpkin, nutmeg and chilli and a few isolates infected cardamom in vitro. No symptoms were observed in vanilla, coconut and arecanut.

5.1. Introduction

Black pepper is one among the spice crops that has been widely cultivated across the world as it is used in medicine and as a condiment. Apart from adverse climatic changes, the cultivation has declined to one-third due to the devastating disease of black pepper called foot rot (Hema *et al.,* 2007) caused by *Phytophthora capsici* and *P. tropicalis* (Bhai *et al.,* 2022; Jeevalatha *et al.,* 2021). Reports say that foot rot disease is among the prime reasons for the decline in black pepper production to one-third. The production constraints of black pepper are a serious matter of concern as it is one of the highly valued commercially important crops and due to its prime value in the global market. Foot rot disease is epiphytotic under favourable conditions, as *Phytophthora* can inflict detrimental harm to its host.

Phytophthora is an oomycete soil-borne pathogen, and its members are both homothallic and heterothallic. *Phytophthora* mainly adopts water as a medium for its easy propagation and remains persistent as either asexual or sexual spores in the field. The better adaptability of the pathogen towards the adverse environmental changes were often attributed to inter-specific hybridization, genetic recombination by sexual reproduction (Donahoo & Lamour 2008), parasexual recombination (Gu *et al.,* 2000), hyphal anastomosis (Stephenson *et al.,* 1974) and migration which ultimately adds to the genetic variability, changes in the virulence pattern of the pathogen and the emergence of fungicide resistant isolates (Pereira *et al.,* 2020). Disclosing the genetic configuration at the molecular level paved the way for enhanced comprehension about the pathogen and further designing better management measures (Burdon, 1993).

Different approaches to study the genetic diversity includes mtDNA analysis (Wang *et al.,* 2009), RAPD (Fu *et al.,* 2003), AFLP (Markert *et al.,* 2010), isozyme analysis (El-Esawi *et al.,* 2017), SNP marker (Jara-Arancio *et al.,* 2022), RAMS and REP-PCR analyses (Truong *et al.,* 2010). The precise reproducibility of RAMS and REP-PCR impelled the present study to employ its benefits to analyze the genetic diversity. The technique RAMS (random amplified microsatellites) was developed initially by Zietkiewicz *et al.* (1994) and Hantula *et al.* (1996) has successfully

implemented this technique in fungi to study its genetic diversity. RAMS is the combination of SSR (simple sequence repeats) and RAPD (Random Amplified Polymorphic DNA) techniques, which intend to increase the specificity and to overcome the setbacks possessed by the individual techniques. Short intergenic repeated sequence, such as REP (Repetitive Extragenic Palindromic) and ERIC (Enterobacterial Repetitive Intergenic Consensus) fingerprint region was identified initially in *Escherichia coli* (Shirzad-Aski & Tabatabaei*,* 2016) and *Salmonella typhimurium.* These can also be employed to study the genetic diversity of closely related strains as unique fingerprints were observed in the PCR products (de Bruijn, 1992).

Box elements composed of combinations of boxA, boxB and boxC subunits, which have the length of 59, 45 and 50 nucleotides (Bilung *et al.,* 2018; Mishra *et al.,* 2015), and have a role in transcription regulation (Borba *et al.,* 2020). REP, ERIC, BOX and M13 were effectively employed in *Phytophthora* species to study its genetic diversity (Masyahit *et al.,* 2019; Truong *et al.,* 2012).

In black pepper, two species of *Phytophthora* have been reported to be associated with foot rot disease in India to date, which are *P. capsici* and *P. tropicalis* (Bhai *et al.,* 2022; Jeevalatha *et al.,* 2021). The present study aims to look into the genetic diversity of the aforementioned *Phytophthora* species using RAMS and REP-PCR fingerprinting. Moreover, the ability of the pathogen to infect other crops has also been assessed.

5.2. Materials and methods

5.2.1. Sources of *Phytophthora* cultures

A total of 48 isolates, with an equal proportion of each species i.e., *P. capsici* and *P. tropicalis* were used in the study. The *Phytophthora* isolates were obtained from the National Repository of *Phytophthora,* ICAR-Indian Institute of Spices Research, Kozhikode, India. The isolates collected from the year 1997 to 2021 from major black pepper growing areas like Kerala, Karnataka, Tamil Nadu and Goa were selected. The isolates were selected in such a manner that each isolate represents

different year (Table 5.1) and place of collection (Fig. 5.1). Carrot agar (Brasier, 1967) medium was used for culturing the isolates and the mycelial discs were stored in the vials containing sterile distilled water for long-term storage. The cultures were grown and/or maintained at 25 ± 1 °C.

Fig. 5.1. Map of the Southern part of India showing the place of collection of *Phytophthora* isolates

Table 5.1. Lists of *P. capsici* and *P. tropicalis* isolates selected for the present study

Sl. No.	Name of species	Isolate	Isolation	Place of collection	State/UT
1	*Phytophthora capsici*	98-81	Black pepper leaf	Kalpakancherry, Malappuram	Kerala
2		98-164	Black pepper leaf	Kargode, Sirsi, Uttara Kannada	Karnataka
3		00-42	Black pepper	Sirsi, Uttara Kannada	Karnataka
4		01-04	Black pepper stem	Adivaram, Kozhikode	Kerala
5		02-20	Rhizosphere soil	Silent Valley, Palakkad	Kerala
6		05-06	Black pepper spike	Peruvannamuzhi, Kozhikode	Kerala
7		05-14	Black pepper leaf	Appangala, Kodagu	Karnataka
8		05-19	Rhizosphere soil	Chettalli, Kodagu	Karnataka
9		06-12	Black pepper leaf	Irikkur, Kannur	Kerala
10		07-03	Black pepper leaf	Chelavoor, Kozhikode	Kerala
11		07-06	Black pepper leaf	Wayanad	Kerala
12		08-01	Black pepper root	Peruvannamuzhi, Kozhikode	Kerala
13		10-02	Black pepper leaf	Bommanahally, Bangalore	Karnataka
14		11-13	Black pepper leaf	Kargunda, Kodagu	Karnataka
15		11-16	Black pepper leaf	Cherambane, Kodagu	Karnataka
16		13-17	Rhizosphere soil	Meppadi, Wayanad	Kerala
17		13-21	Black pepper leaf	Adimali, Idukki	Kerala
18		13-41	Black pepper leaf	Madikai, Kasaragod	Kerala
19		13-42	Black pepper leaf	Madikai, Kasaragod	Kerala
20		13-48	Black pepper root	Goa	Goa
21		18-12	Black pepper leaf	Idukki	Kerala
22		20-05	Black pepper leaf	Panniyur, Kannur	Kerala
23		20-06	Black pepper leaf	Ambalavayal, Wayanad	Kerala
24		21-01	Black pepper leaf	Panniyur, Kannur	Kerala
25	*Phytophthora tropicalis*	97-19	Black pepper stem	Kottakkal, Malappuram	Kerala
26		97-55	Black pepper root	Valparai, Coimbatore	Tamil Nadu

27	98-65	Black pepper leaf	Valnoor, Kodagu	Karnataka
28	98-93	Black pepper leaf	Adyanadka, Dakshin Kannada	Karnataka
29	98-175	Black pepper root	Isloor, Sirsi, Uttara Kannada	Karnataka
30	99-136	Black pepper leaf	Silver Cloud, Wayanad	Kerala
31	00-38	Black pepper	Sidapur, Kodagu	Karnataka
32	01-20	Black pepper	Silent Valley, Palakkad	Kerala
33	03-07	Black pepper leaf	Adivaram, Kozhikode	Kerala
34	05-17	Black pepper leaf	Chettalli, Kodagu	Karnataka
35	06-11	Black pepper berries	Edayannur, Iritti, Kannur	Kerala
36	06-17	Black pepper leaf	Rasi Estate, Yercaud, Salem	Tamil Nadu
37	08-07	Black pepper stem	Koorachundu, Kozhikode	Kerala
38	09-01	Black pepper stem	Peruvannamuzhi, Kozhikode	Kerala
39	09-18	Rhizosphere soil	Pamathatt, Kasaragod	Kerala
40	09-25	Rhizosphere soil	Kanjirapuzha, Palakkad	Kerala
41	11-20	Black pepper leaf	Sunnapulikote, Kodagu	Karnataka
42	11-29	Rhizosphere soil	Wayanad	Kerala
43	11-30	Rhizosphere soil	Wayanad	Kerala
44	13-23	Rhizosphere soil	Nedumkandam, Idukki	Kerala
45	13-33	Rhizosphere soil	Kumali, Idukki	Kerala
46	13-45	Black pepper leaf	Pilicode, Kasaragod	Kerala
47	13-53	Black pepper root	Goa	Goa
48	16-06	Black pepper leaf	Appangala	Karnataka

5.2.2. DNA extraction and species identification

The 3-day-old mycelial discs were inoculated into Ribeiro's broth (Erwin & Ribeiro 1996). On the fourth day, the actively growing mycelia were then taken out from the broth and placed onto blotting paper to remove the broth that had been trapped within the mycelia. The DNA extraction protocol was adopted from Sheji *et al*. (2009) with slight modifications (Jeevalatha *et al.,* 2021). 100 mg of the dried mycelia was ground by adding a pinch of glass wool powder and 1 mL of STE extraction buffer. The cell debris was removed after centrifugation at 12,000 rpm for 10 min. 500 µl of tris phenol, chloroform, and isoamyl alcohol (25:24:1) was added, mixed by gentle inversion and centrifuged at 12,000 rpm for 10 min. To the aqueous layer, an equal volume of chloroform, isoamyl alcohol (24:1) was added and inverted gently and centrifuged at 12,000 rpm for 10 min. 0.6 V of isopropanol was added to the aqueous layer to precipitate the DNA. The DNA was washed with 70% ethanol and dissolved in nuclease-free water. The quality of the DNA was measured using DS-11+ Spectrophotometer (DeNovix®). Species identity was analyzed by PCR assay using Ypt1 gene-based species-specific primers (Jeevalatha *et al.,* 2021). The results were compared with the results of ITS rDNA sequencing and ITS-RFLP of *Phytophthora* species infecting black pepper, which were reported by earlier reports of Bhai *et al*. (2022).

5.2.3. RAMS and REP-PCR analyses

The particulars of primers used for RAMS and REP-PCR analyses are given in Table 5.2. PCR reaction mix (20 µL) was set up in a 200 µL flat cap PCR tube. To a PCR tube, 10 µL of Emerald Amp GT PCR Master Mix (2X Premix; Takara Bio Inc, Shiga, Japan) was added, followed by 0.8 µM of primer and 20 ng of DNA. PCR was carried out in a Gradient Thermal Cycler IG-96GEP (iGene Labserve, New Delhi, India), by following the thermal cycling conditions described by Hantula *et al.* (1996). The PCR products were run in 2.5% agarose gel in 1X TAE buffer at 80 V for 3 h and visualized under a UV transilluminator. A volume of 25 µL reaction was set up for the PCR amplification for REP-PCR analysis. The reaction mixture was composed of 12.5 µL of Emerald Amp GT PCR Master Mix (2X Premix;

Takara Bio Inc., Shiga, Japan), 0.4 μM primer and 20 ng DNA template. The temperature profile of thermal cycling is given in Table 5.2 and was performed in a Gradient Thermal Cycler IG-96GEP (iGene Labserve, New Delhi, India).

5.2.4. Analysis of genetic diversity

PCR-generated bands were visualized in agarose gel images of RAMS and REP-PCR and scored either present (1) or absent (0). Only those bands which are strong and reproducible were scored. Genetic diversity studies were performed with RAMS and REP-PCR generated data individually and also by combining the scoring output of both analyses. The software NTSYSpc Version 2.10e (Rohlf, 2000) was used to execute Unweighted Pair-Group Method using Arithmetic Averages (UPGMA) clustering using DICE similarity coefficient. POPGENE version 1.32 (Yeh *et al.,* 1997) was used to compute the genetic diversity indices. GenAlEx 6.5 software with default variable settings (Peakall & Smouse, 2012) was used for the principal coordinate analysis (PCoA), the binary data obtained from RAMS, REP-PCR and integrated RAMS & REP-PCR analyses as the input.

5.2.5. Morphological characteristics of the colony and sporangia

Further studies were carried out with a total of sixteen representative isolates from each of the sub-clusters obtained by RAMS and REP-PCR data generated UPGMA dendrogram. The selected isolates were cultured in carrot agar media and were incubated at 25 ± 1°C. The pattern of growth was observed and photographed on the fifth day after inoculation. The sporangia production was induced by placing the 5 mm discs of actively growing mycelia on a Petri plate containing sterile distilled water and incubated at 25±1°C under continuous illumination for 48 h. Leica DM 5000 B microscope (Leica Mikrosystems Vertrich GmbH, Germany) was used to check and photograph the sporangia.

Table 5.2. Details of primers used and the PCR cycle conditions followed for RAMS and REP-PCR

Primer	Sequence	Temperature cycle
RAMS (Hantula *et al.,* 1996)		
CCA	5'DD$(CCA)_5$	95°C 10 min, 95°C 30 s, 64°C 45 s, 72°C 2 min, 72°C 7 min- 35 cycles
GT	5'YHY$(GT)_5$G	95°C 10 min, 95°C 30 s, 53°C 45 s, 72°C 2 min, 72°C 7 min- 35 cycles
ACA	5'BDB$(ACA)_5$	95°C 10 min, 95°C 30 s, 49°C 45 s, 72°C 2 min, 72°C 7 min- 35 cycles
CGA	5'DHB$(CGA)_5$	95°C 10 min, 95°C 30 s, 60°C 45 s, 72°C 2 min, 72°C 7 min- 35 cycles
REP-PCR (Masyahit *et al.,* 2019)		
BOX1A	5′-CTACGGCAAGGCGACGCTGACG-3′	95°C 7 min, 94°C 1 min, 53°C 1 min, 65°C 8 min, 65°C 16 min- 30 cycles
ERIC1R	5′-ATGTAAGCTCCTGGGGATTCAC-3′	
ERIC2	5′-AAGTAAGTGACTGGGGTGAGCG-3′	95°C 7 min, 94°C 1 min, 52°C 1 min, 65°C 8 min, 65°C 16 min- 30 cycles
REP1R-I	5′-IIIICGICGICATCIGGC-3′	94°C 7 min, 94°C 1 min, 40°C 1 min, 65°C 8 min, 65°C 16 min- 35 cycles
REP2-I	5′-ICGICTTATCIGGCCTAC-3′	
M13	5′-GAGGGTGGCGGTTCT-3′	93°C 3 min, 93°C 1 min, 55°C 1 min, 72°C 1 min, 72°C 10 min- 45 cycles

5.2.6. Infectivity analysis

Infectivity analysis was performed with sixteen isolates with equal proportion of each species i.e., *P. capsici* and *P. tropicalis* representing different sub-clusters from the above results. The analysis was conducted on the host plant black pepper and other crops such as pumpkin, arecanut, tomato, vanilla, cardamom, cucumber, chilli, coconut and nutmeg using the detached fruit/leaf inoculation method *in vitro*. Discs of actively growing mycelia (5 mm) of the isolates were inoculated either on the abaxial surface of the leaf or on the surface of the fruit of the selected crops. The carrot agar discs without the mycelium were used as a control for inoculation and were incubated at 25±1°C in a moist chamber. The analysis was performed in triplicate. These were regularly monitored for the development of symptoms, and the lesion diameter was recorded and the leaf/fruit was photographed at 48 h after inoculation. The area of the lesion was computed using the formula (da Silva *et al.,* 2012) given below.

$S = \pi (L \times W) / 4$

where S is the surface area of the lesion, L is the length of lesion and W is the width of lesion. R Studio R version 4.2.1 (R Core Team 2022) was used to perform ANOVA.

5.3. Results

5.3.1. Species identification

The species identity of the selected isolates was analyzed by the PCR assay using the species-specific primers and confirmed by comparing the ITS rDNA sequencing and ITS-RFLP assay results (Bhai *et al.,* 2022).

5.3.2. RAMS and REP-PCR analyses

The agarose gel images of RAMS and REP-PCR analyses were depicted in Fig. 5.2. The outcome of RAMS analysis among the 48 isolates revealed a total of 71 loci of which greater polymorphism was observed in isolates from Kerala which is 88.73% followed by Karnataka (83.1%), Tamil Nadu (11.27%) and Goa (49.3%). The

combined data of all states showed 90.14% of polymorphic loci. Regarding the REP-PCR analysis, a total of 89 loci were revealed, and 96.63% of the polymorphisms were identified in isolates of Kerala, Karnataka accounted for 78.65%, Tamil Nadu for 8.9%, and Goa for 41.57% of polymorphisms. The analysis of REP-PCR data of all states, when collated showed about 96.63% of the polymorphic loci. When the data from both analyses were combined, the results generated showed that among the isolates from all the states, a total of 160 loci and 93.75% were polymorphic. The Shannon diversity index of Kerala and Karnataka populations were 0.4414 and 0.4100, respectively, and Nei's gene diversity of Kerala and Karnataka populations were 0.2892 and 0.2717, respectively (Table 5.3). The diversity parameters were also computed separately for each primer (Table 5.4), and the outcome showed that among the RAMS primers, greater polymorphism was observed for GT (100%) and least for the ACA primer (80.95%). On the other hand, REP-PCR yielded 100% polymorphisms for the primers BOX1, M13 and Rep-1R& Rep-2I with number of loci 28, 21 and 23 respectively. Whereas, 82.35% polymorphic loci were observed for ERIC1R & ERIC2 primers. UPGMA dendrogram generated using the binary input of RAMS and REP-PCR depicted in Fig. 5.3 clearly grouped the two species i.e., *P. capsici* and *P. tropicalis* into two separate clusters broadly and were further split into four sub-clusters I, II, III & IV. All the *P. capsici* isolates were in sub-cluster I & II, and the *P. tropicalis* isolates were grouped in sub-cluster III & IV. The distribution of the isolates collected from different states was random, and hence the correlation between the cluster and the geographical origin of the isolates cannot be drawn. The dendrogram also shows that the similarity among the isolates, which were grouped closer was up to 97 %, indicating that the population is heterogeneous and the isolates were unique. But when we compare RAMS and REP-PCR techniques, REP-PCR alone was found to be efficient in grouping the isolates into four different clusters, with each species being clearly differentiated, rather than RAMS analysis (Fig. 5.4), where slight variations were observed i.e., sub-cluster II where *P. capsici* was grouped (with three of the isolates), formed a major cluster with III & IV sub-clusters; besides that, cluster topology was maintained in the RAMS dendrogram too.

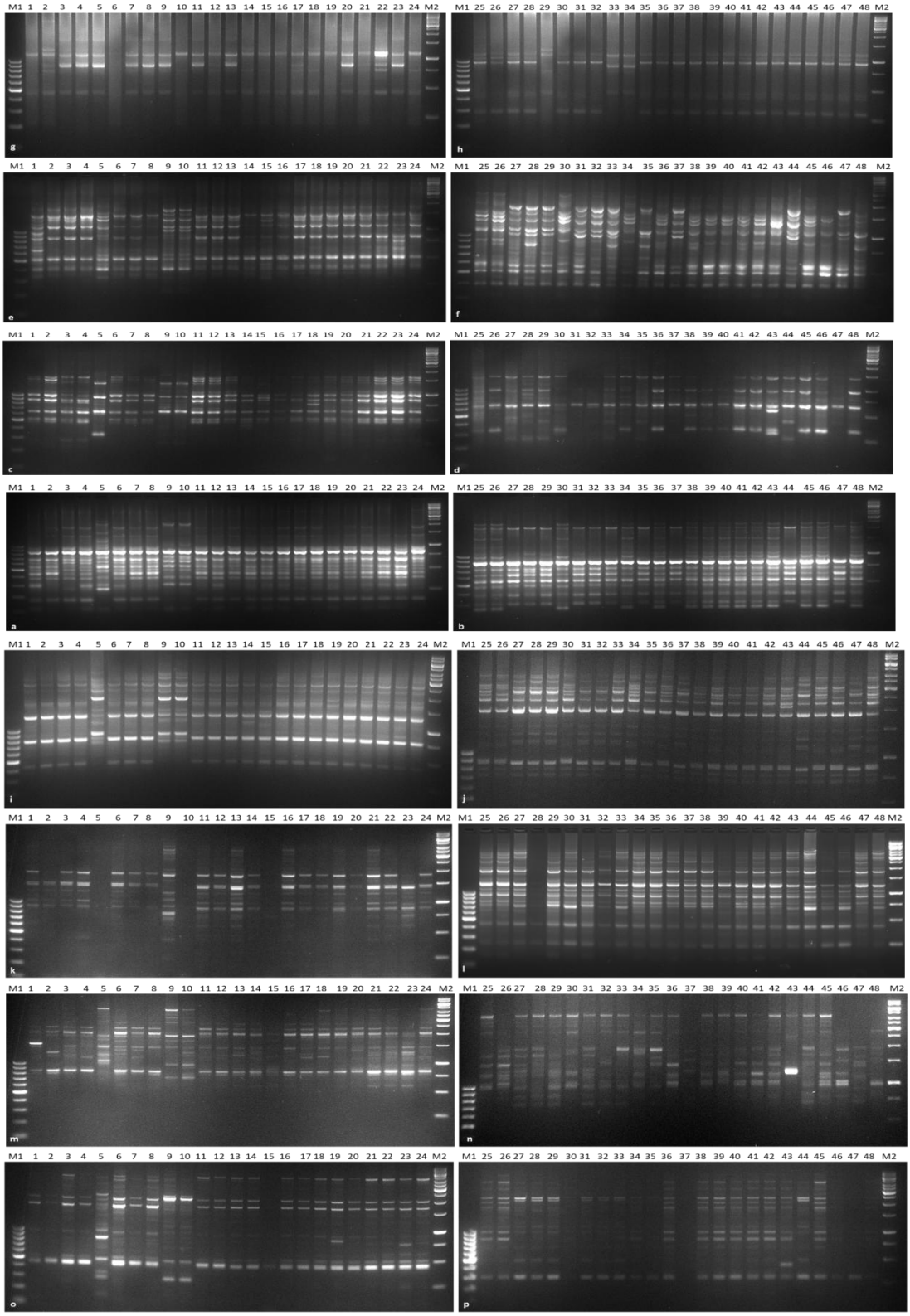


Fig. 5.2. Agarose gel electrophoresis of RAMS & REP-PCR. a-h) RAMS: ACA, CCA, CGA, GT; i-p) REP: BOX1, ERIC, M13, Rep-1R Rep-2I. Lane M1 - 100bp ladder, lanes 1-24 *P. capsici* isolates, lanes 25-48 *P. tropicalis* isolates and lane M2 - 1kb ladder

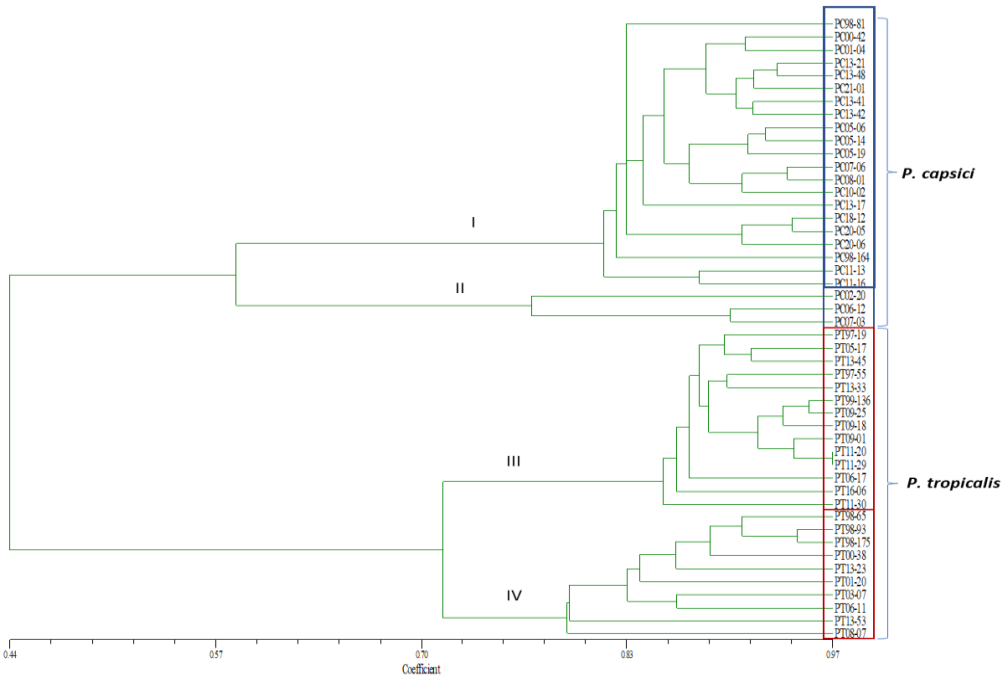


Fig. 5.3. UPGMA dendrogram based on the RAMS and REP-PCR data of 48 *Phytophthora* isolates

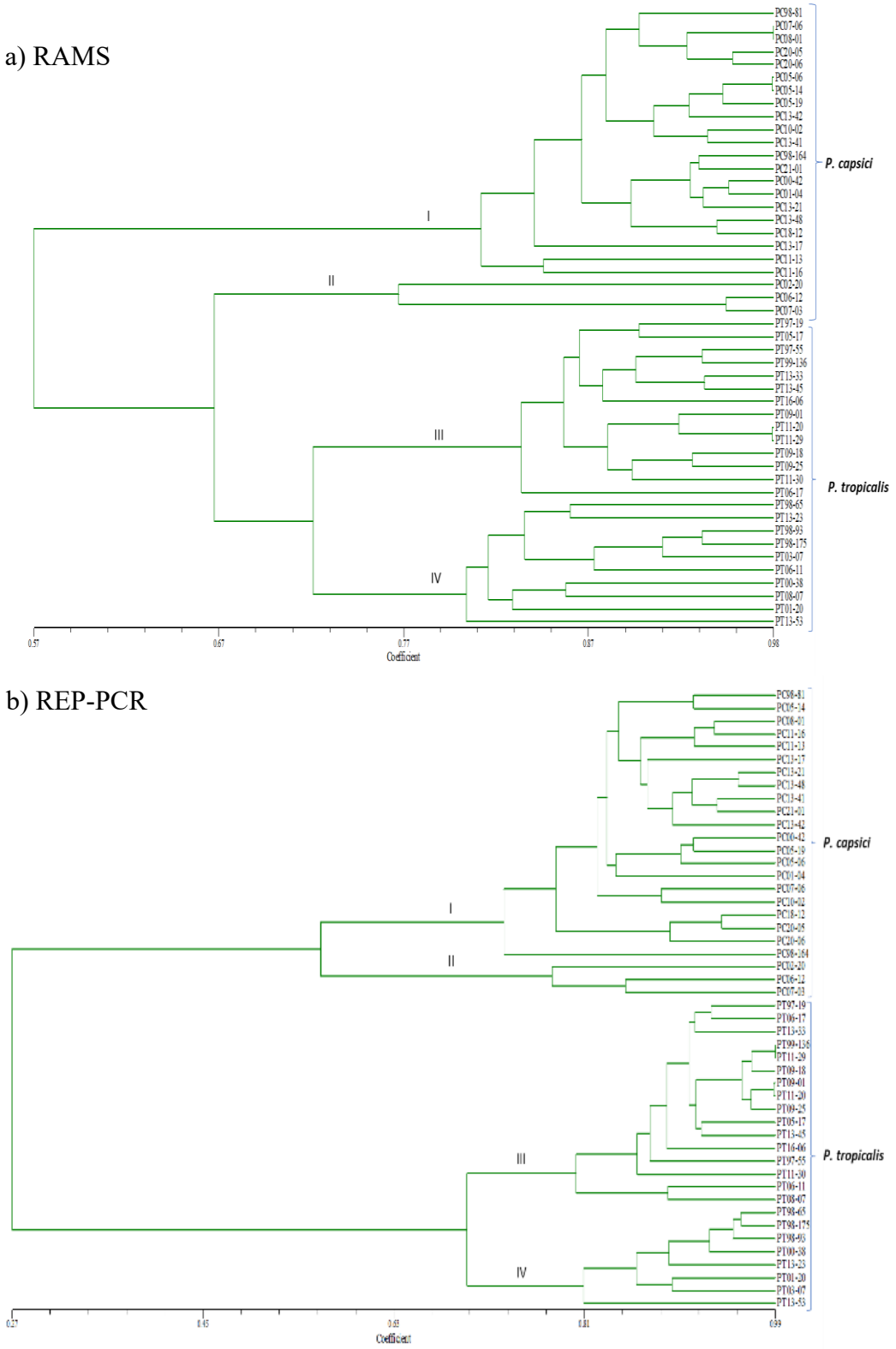


Fig. 5.4. UPGMA dendrogram of selected isolates a) RAMS data, b) REP-PCR data

Table 5.3. The diversity parameters of 48 *Phytophthora* isolates as computed using POPGENE version 1.32

Marker	No. of isolates	Total no. Loci	No. of polymorphic loci (% of total)	Nei gene diversity (h)	Shannon diversity index (I)	Population group*
RAMS						
Combined (all states)	48	71	64 (90.14)	0.3204	0.4751	I, II, III, IV
Kerala	30	71	63 (88.73)	0.3128	0.4654	I, II, III, IV
Karnataka	14	71	59 (83.10)	0.3124	0.4605	I, III, IV
Tamil Nadu	2	71	8 (11.27)	0.0467	0.0681	III
Goa	2	71	35 (49.30)	0.2042	0.2981	I, IV
REP						
Combined (all states)	48	89	86 (96.63)	0.2660	0.4158	I, II, III, IV
Kerala	30	89	86 (96.63)	0.2704	0.4223	I, II, III, IV
Karnataka	14	89	70 (78.65)	0.2392	0.3698	I, III, IV
Tamil Nadu	2	89	8 (8.99)	0.0372	0.0544	III
Goa	2	89	37 (41.57)	0.1722	0.2514	I, IV
RAMS + REP						
Combined (all states)	48	160	150 (93.75)	0.2901	0.4421	I, II, III, IV
Kerala	30	160	149 (93.12)	0.2892	0.4414	I, II, III, IV
Karnataka	14	160	129 (80.62)	0.2717	0.4100	I, III, IV
Tamil Nadu	2	160	16 (10.00)	0.0414	0.0605	III
Goa	2	160	72 (45.00)	0.1864	0.2721	I, IV

*Population group I and II – *P. capsici*, III and IV – *P. tropicalis*

Table 5.4. The diversity parameters of 48 *Phytophthora* isolates identified by each of the primers

Marker diversity	Total No. loci	No. of polymorphic loci (% of total)	Nei gene diversity (h)	Shannon index (I)
RAMS				
ACA	21	17(80.95)	0.33	0.47
CCA	20	19(95)	0.28	0.43
CGA	17	15(88.24)	0.39	0.56
GT	13	13(100)	0.28	0.44
REP-PCR				
BOX1	28	28(100)	0.28	0.44
M13	21	21(100)	0.27	0.42
Rep-1R & Rep-2I	23	23(100)	0.27	0.43
ERIC1R & ERIC 2	17	14(82.35)	0.21	0.33

5.3.3. Principal coordinate analysis (PCoA)

PCoA analysis was implemented for the data generated using each of RAMS and REP-PCR analyses, and also by collating both analyses based on the distance matrix using data standardization. The topology of the scatter plot obtained by PCoA analysis was similar to that of the UPGMA dendrogram, which grouped the 48 isolates into four clusters, viz. cluster I & II, which consist of *P. capsici* isolates denoted by blue circles and cluster III & IV with *P. tropicalis* isolates denoted by green circles (Fig. 5.5). PCoA analysis for RAMS data, the cumulative variation of the first three coordinates explicated was about 76.40 %, and there was a variation in the topology between the UPGMA dendrogram and PCoA. The topology was found to be varied only in the case of *P. capsici* isolates. Whereas PCoA analysis using REP-PCR data, the cumulative variation of the first three coordinates explained was 87.58%, and the topology of UPGMA and PCoA analyses were similar (Fig. 5.6).

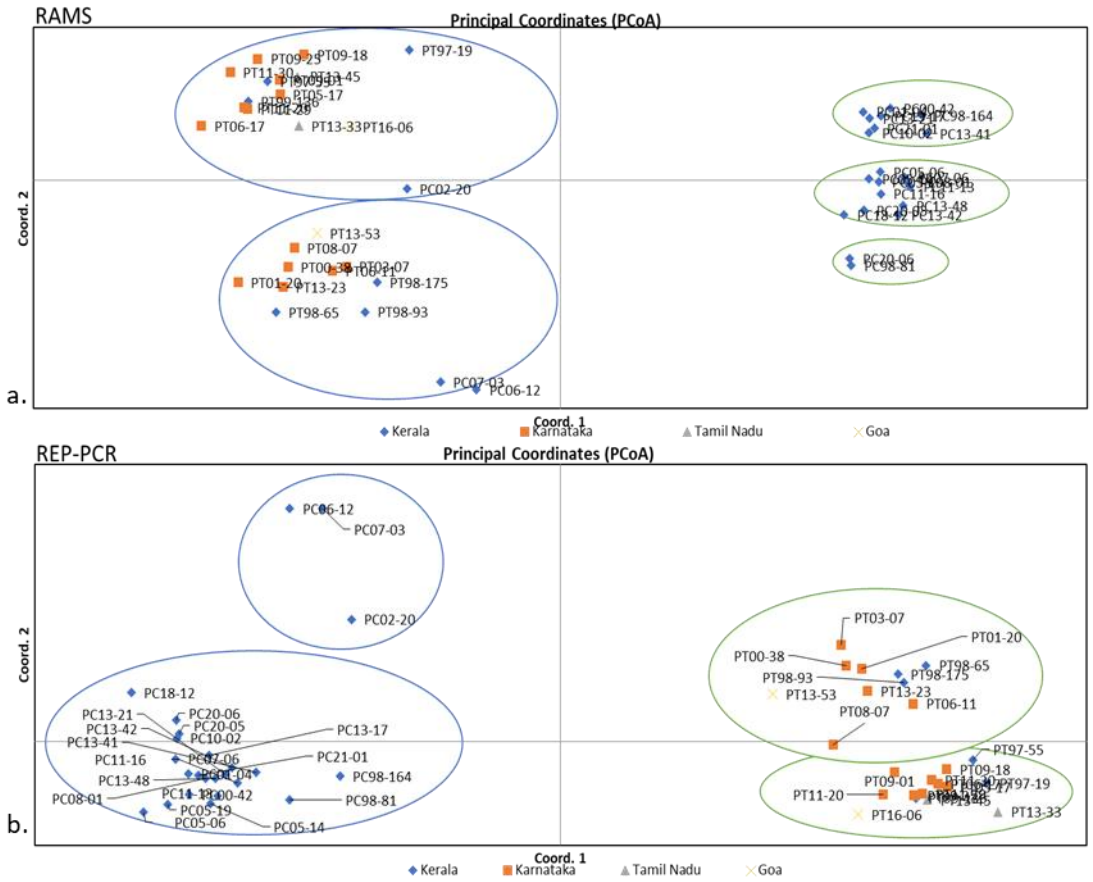


Fig. 5.5. Principal Coordinate Analysis (PCoA) of a) RAMS; b) REP-PCR data of the selected 48 isolates

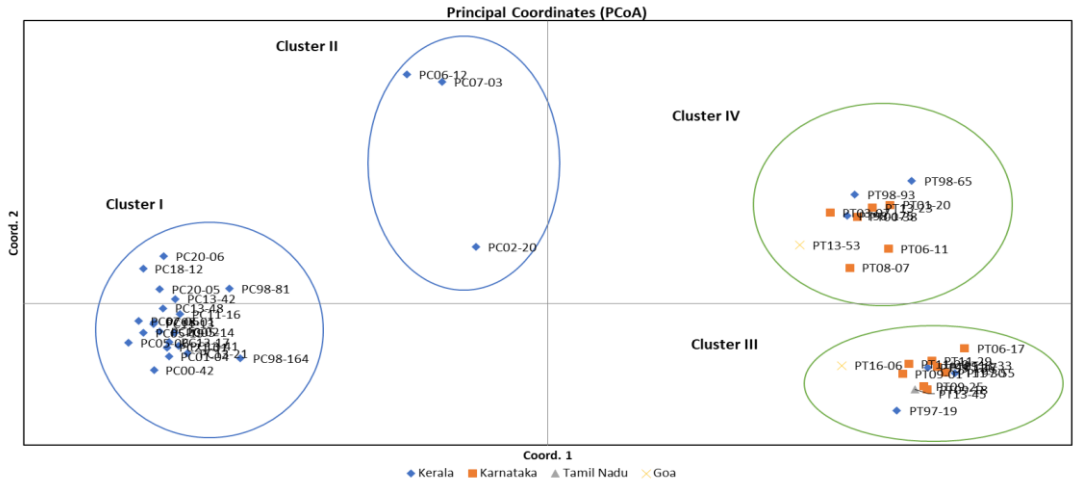


Fig. 5.6. Principal Coordinate Analysis (PCoA) of combined data of RAMS and REP-PCR depicting the four cluster

5.3.4. Morphological and sporangial characters

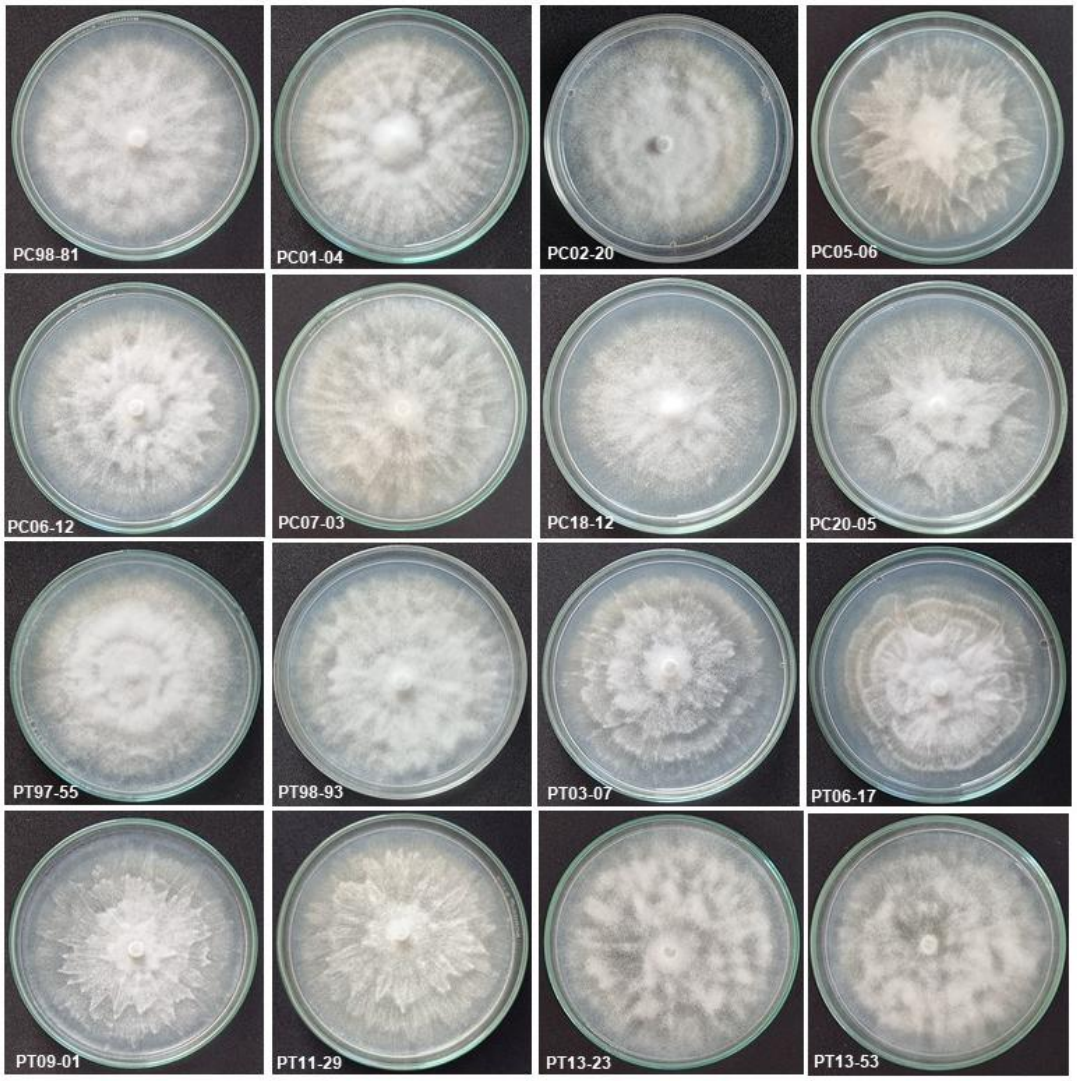


Fig. 5.7. Petri plate with carrot agar medium displaying the colony morphology of *Phytophthora* isolates

Studies on colony morphology of the sixteen isolates showed that the isolates displayed varied patterns namely stellate with cottony mycelium (PT13-23 and PT13-53), modified chrysanthemum (PC98-81, PC05-06, PC07-03 and PT98-93), stellate (PC20-05), modified stellate (PC06-12, PC18-12, PT03-07, PT09-01 and PT11-29), cottony with concentric rings (PC02-20), stellate with concentric rings (PC01-04) and modified petaloid (PT06-17) (Fig. 5.7). The microscopic observation showed variety of sporangial shapes such as obpyriform, ovoid-obpyriform, pyriform, limoniform, ovoid, globose, elongated, elliptical and naviculate (Fig. 5.8).

No correlation was observed between the colony morphology, sporangial, shape and clustering for the isolates.

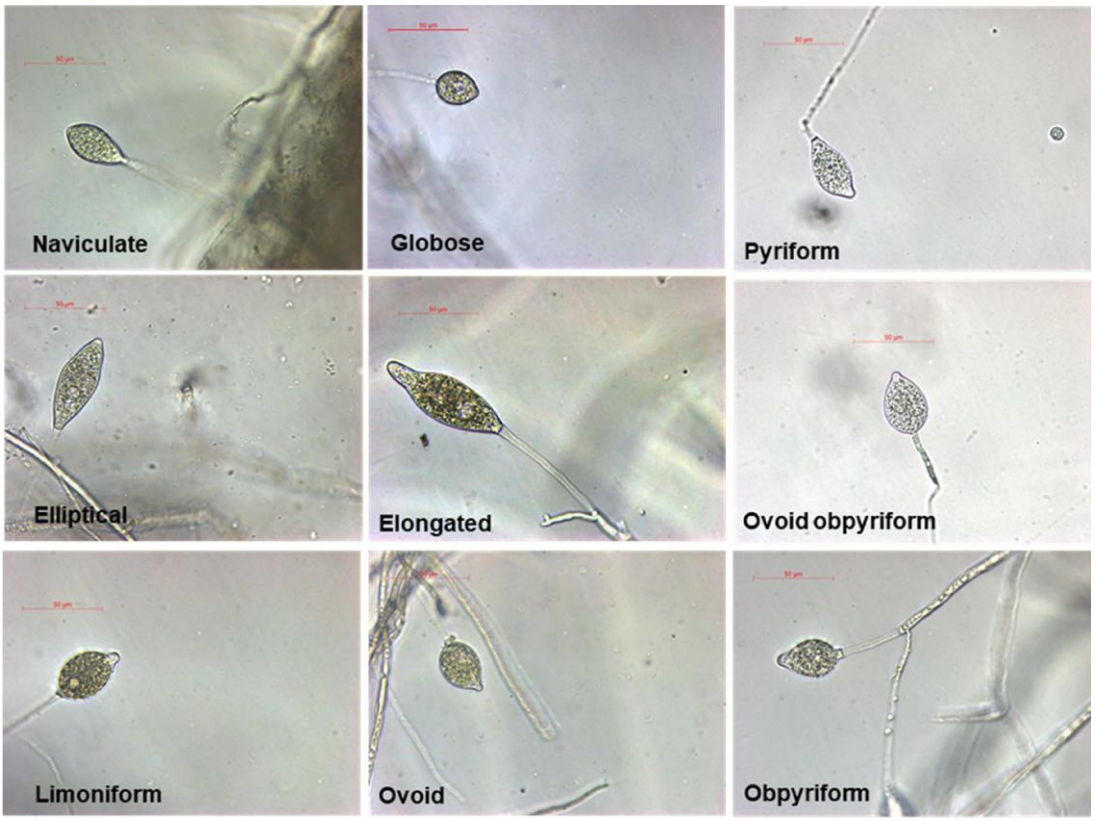


Fig. 5.8. Microscopic images of selected *Phytophthora* isolates showing different shapes of sporangia

5.3.5. Infectivity analysis

All the 16 selected isolates from black pepper infected crops like pumpkin, cucumber, tomato and nutmeg and few isolates such as, PC20-05, PC06-12, PT13-23 and PT06-17 infected cardamom (Fig. 5.9). The typical characteristics of the lesion produced by the *Phytophthora capsici* and *P. tropicalis* on black pepper leaves includes water-soaked lesion with fimbriate margins and dark brown lesion with yellow hallow respectively. On nutmeg leaves, black pepper isolates produced a dark brown lesion which progressed to the entire leaf surface over time. Water-soaked lesion was observed at the initial stage and later on, white cottony mycelial growth appeared on the lesion. The lesion on tomato leaves appeared as a light brownish lesion and on pumpkin, there were sunken spots with water-soaked

lesions. The lesion on the cardamom leaf displayed a brown colour with yellow margin. Concerning the area of lesion produced by the isolates on black pepper, the largest area of lesion was inflicted by the isolate PT97-55 with the lesion area of 10.54 cm^2 followed by the isolate PC06-12 with 8.82 cm^2. Isolate PC98–81 (area of lesion 0.45 cm^2) was the least virulent. In case of nutmeg, a comparable pattern of virulence was displayed by the isolates PC01-04, PT13-53 and PT13-23 with areas of lesion ranging from 10.33 cm^2, 10.46 cm^2 to 10.71 cm^2, and PC18-12 was least virulent (area of lesion 0.77 cm^2). Significant difference among the areas of lesion was not observed in tomato, as it ranged from 0.41 cm^2 to 2.58 cm^2. In chilli, the isolates PT13-23, PT06-17, PC07-03 and PT03-07 were highly virulent with the lesion diameter 14.52 cm^2, 14.01 cm^2, 13.86 cm^2 and 13.73 cm^2 respectively. The isolates PT09-01 and PC98-81 were least virulent, with areas of lesion 0.79 cm^2 and 0.64 cm^2.

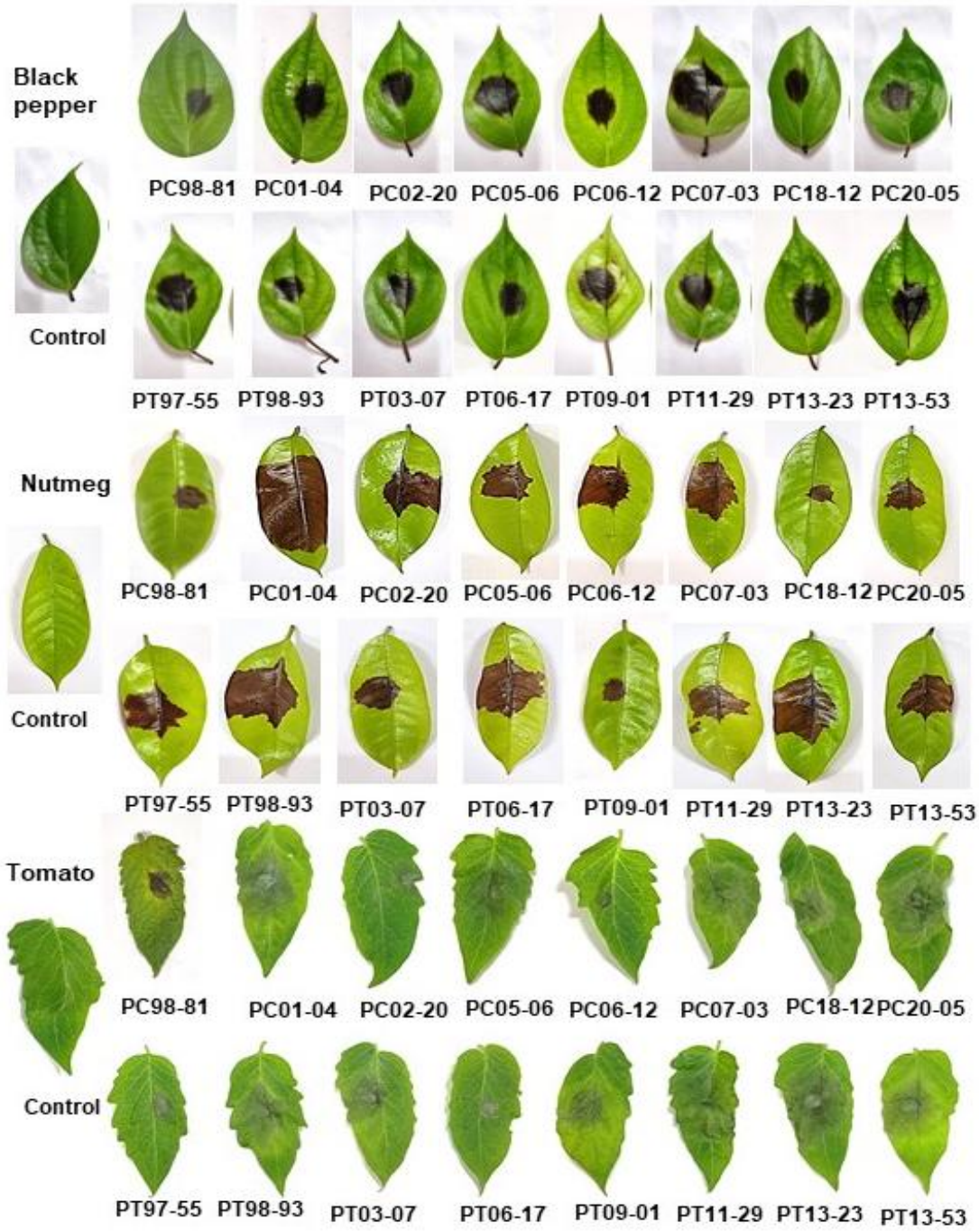
The isolate PT13-53 was highly virulent in pumpkin, which produced a lesion diameter of 9.94 cm^2 followed by PT06-17 with a lesion diameter of 6.75 cm^2, the isolate PT97-55 was found to be least virulent with a lesion diameter of 0.82 cm^2. Interestingly, it was observed that only a few of the isolates from the sixteen isolates, namely PC05-06, PT06-17, PT13-23 and PC20-05 infected cardamom with a lesion diameter ranging from 0.21 to 0.77 cm^2. Table 5.5 shows the ANOVA (Analysis of Variance) of the area of lesion calculated by taking the three replicate readings; the values with the same superscript letters showed a comparable pattern of virulence as compared to the rest of the values. When we consider the pathogenicity of the isolates, isolates PT97-55 and PC06-12 were most aggressive in chilli and black pepper but were meek on other selected crops. Moderately virulent isolates (PC05-06 and PC20-05) on chilli and black pepper were mild on pumpkin and nutmeg. The isolates PC02-20, PT06-17 and PT11-29 were moderately aggressive in nutmeg and black pepper, but in the rest of the crops, PC02-20 was least virulent and PT06-17 and PT11-29 were aggressive in chilli. Although the isolates PC07-03, PC18-12, PT09-01, PT13-23 and PT13-53 displayed moderate level of virulence in black pepper, the isolates PC07-03, PT13-23 and PT13-53 were aggressive in both nutmeg and chilli whereas PT13-53 is also aggressive in

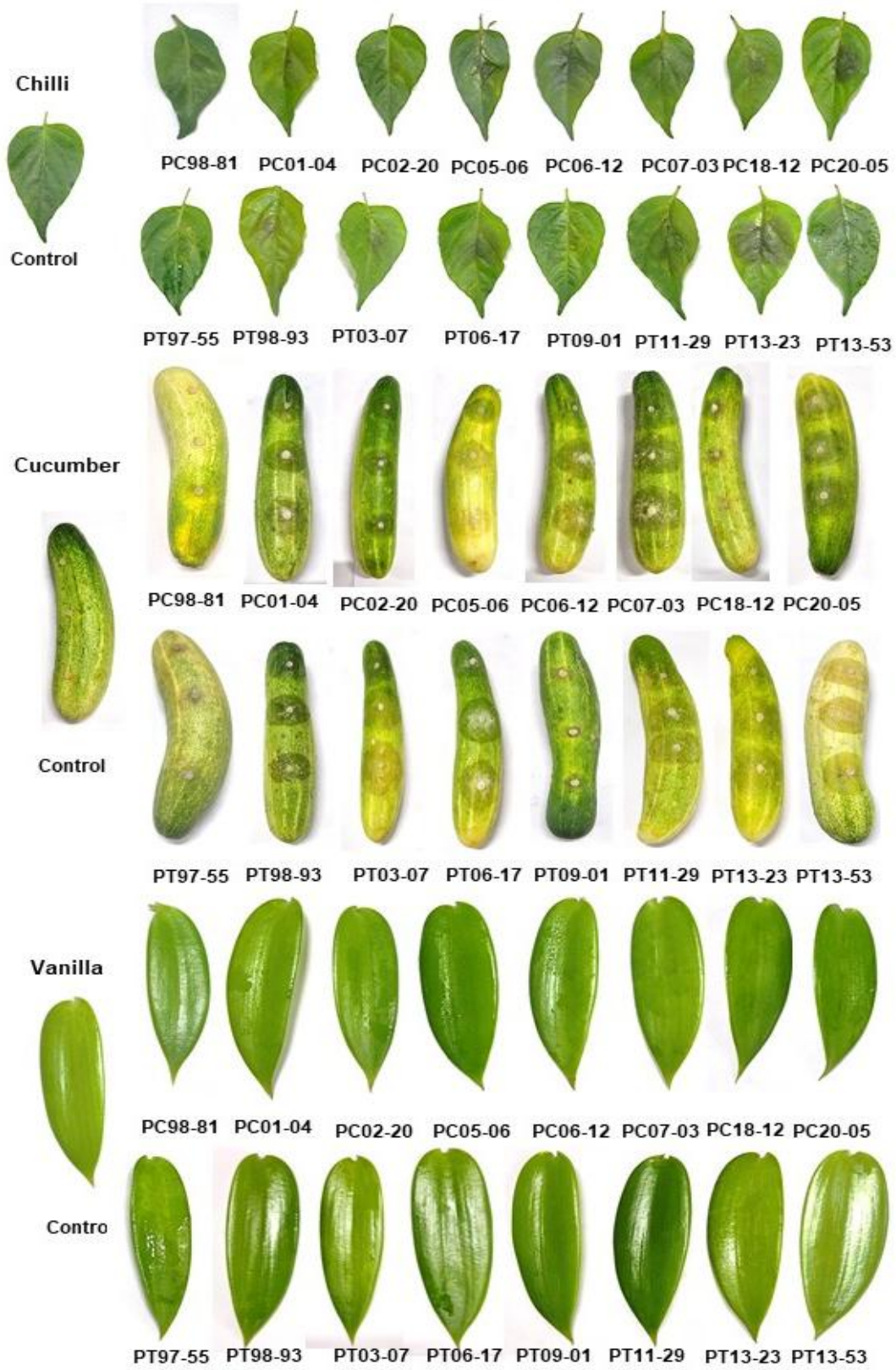
pumpkin. PC98-81, PT98-93, PC01-04 and PT03-07 isolates were less virulent in black pepper, whereas PC01-04 was aggressive in both chilli and nutmeg and moderately virulent was PT98-93. The crops such as arecanut, vanilla and coconut remained uninfected by the black pepper *Phytophthora* isolates (Fig. 5.9). The above observations show that the pattern of virulence among the isolates was random and there was no correlation with the UPGMA cluster.

Table 5.5. ANOVA computed using the area of lesion caused by *Phytophthora* infection on selected crops

Isolates	Black pepper	Nutmeg	Tomato	Chilli	Pumpkin	Cardamom
98-81	0.45 ^{e}	1.84 ^{gh}	1.21 ^{a}	0.64 ^{h}	5.75 ^{abc}	0.00 ^{d}
01-04	3.61 ^{cde}	10.33 ^{a}	1.56 ^{a}	9.09 ^{cd}	2.93 ^{bcd}	0.00 ^{d}
02-20	5.21 ^{bcd}	4.87 ^{cdefg}	0.66 ^{a}	4.63 ^{efg}	1.78 ^{bcd}	0.00 ^{d}
05-06	6.14 ^{bcd}	2.90 ^{efgh}	1.05 ^{a}	5.83 ^{ef}	2.76 ^{bcd}	0.21 ^{c}
06-12	8.82 ^{ab}	6.19 ^{bcde}	0.41 ^{a}	11.05 ^{bc}	2.02 ^{bcd}	0.00 ^{d}
07-03	6.07 ^{bcd}	9.06 ^{ab}	2.58 ^{a}	13.86 ^{ab}	2.31 ^{bcd}	0.00 ^{d}
18-12	4.84 ^{bcde}	0.77 ^{h}	1.53 ^{a}	2.02 ^{gh}	1.16 ^{cd}	0.00 ^{d}
20-05	4.06 ^{cde}	3.59 ^{defgh}	2.46 ^{a}	5.95 ^{ef}	1.89 ^{bcd}	0.37 ^{b}
97-55	10.54 ^{a}	2.17 ^{fgh}	0.66 ^{a}	3.77 ^{fg}	0.28 ^{d}	0.00 ^{d}
98-93	2.75 ^{de}	6.50 ^{bcd}	2.19 ^{a}	7.28 ^{de}	1.63 ^{bcd}	0.00 ^{d}
03-07	3.37 ^{cde}	3.02 ^{efgh}	0.90 ^{a}	13.73 ^{ab}	1.36 ^{cd}	0.00 ^{d}
06-17	5.03 ^{bcd}	7.63 ^{abc}	1.05 ^{a}	14.01 ^{ab}	6.75 ^{ab}	0.77 ^{a}
09-01	6.36 ^{abcd}	2.49 ^{fgh}	2.13 ^{a}	0.7 9 ^{h}	2.05 ^{bcd}	0.00 ^{d}
11-29	5.29 ^{bcd}	5.39 ^{cdef}	1.83 ^{a}	10.26 ^{c}	2.04 ^{bcd}	0.00 ^{d}
13-23	4.66 ^{bcde}	10.71 ^{a}	1.88 ^{a}	14.52 ^{a}	2.28 ^{bcd}	0.22 ^{c}
13-53	7.46 ^{abc}	10.46 ^{a}	1.72 ^{a}	10.44 ^{c}	9.94 ^{a}	0.00 ^{d}
CV	44.78	32.04	126.73	20.79	89.19	91.13
t-value	2.03	2.03	2.03	2.03	2.03	2.04
LSD	3.94	2.92	3.13	2.76	4.35	0.15

Coconut, arecanut and vanilla was not included as there were no infections. Values with the same superscript letters are not significantly different as detected by DMRT ($p<0.05$).


Black pepper
PC98-81
PC01-04
PC02-20
PC05-06
PC06-12
PC07-03
PC18-12
PC20-05
Control
PT97-55
PT98-93
PT03-07
PT06-17
PT09-01
PT11-29
PT13-23
PT13-53
Nutmeg
PC98-81
PC01-04
PC02-20
PC05-06
PC06-12
PC07-03
PC18-12
PC20-05
Control
PT97-55
PT98-93
PT03-07
PT06-17
PT09-01
PT11-29
PT13-23
PT13-53
Tomato
PC98-81
PC01-04
PC02-20
PC05-06
PC06-12
PC07-03
PC18-12
PC20-05
Control
PT97-55
PT98-93
PT03-07
PT06-17
PT09-01
PT11-29
PT13-23
PT13-53


Chilli
PC98-81
PC01-04
PC02-20
PC05-06
PC06-12
PC07-03
PC18-12
PC20-05
Control
PT97-55
PT98-93
PT03-07
PT06-17
PT09-01
PT11-29
PT13-23
PT13-53
Cucumber
PC98-81
PC01-04
PC02-20
PC05-06
PC06-12
PC07-03
PC18-12
PC20-05
Control
PT97-55
PT98-93
PT03-07
PT06-17
PT09-01
PT11-29
PT13-23
PT13-53
Vanilla
PC98-81
PC01-04
PC02-20
PC05-06
PC06-12
PC07-03
PC18-12
PC20-05
Contro
PT97-55
PT98-93
PT03-07
PT06-17
PT09-01
PT11-29
PT13-23
PT13-53

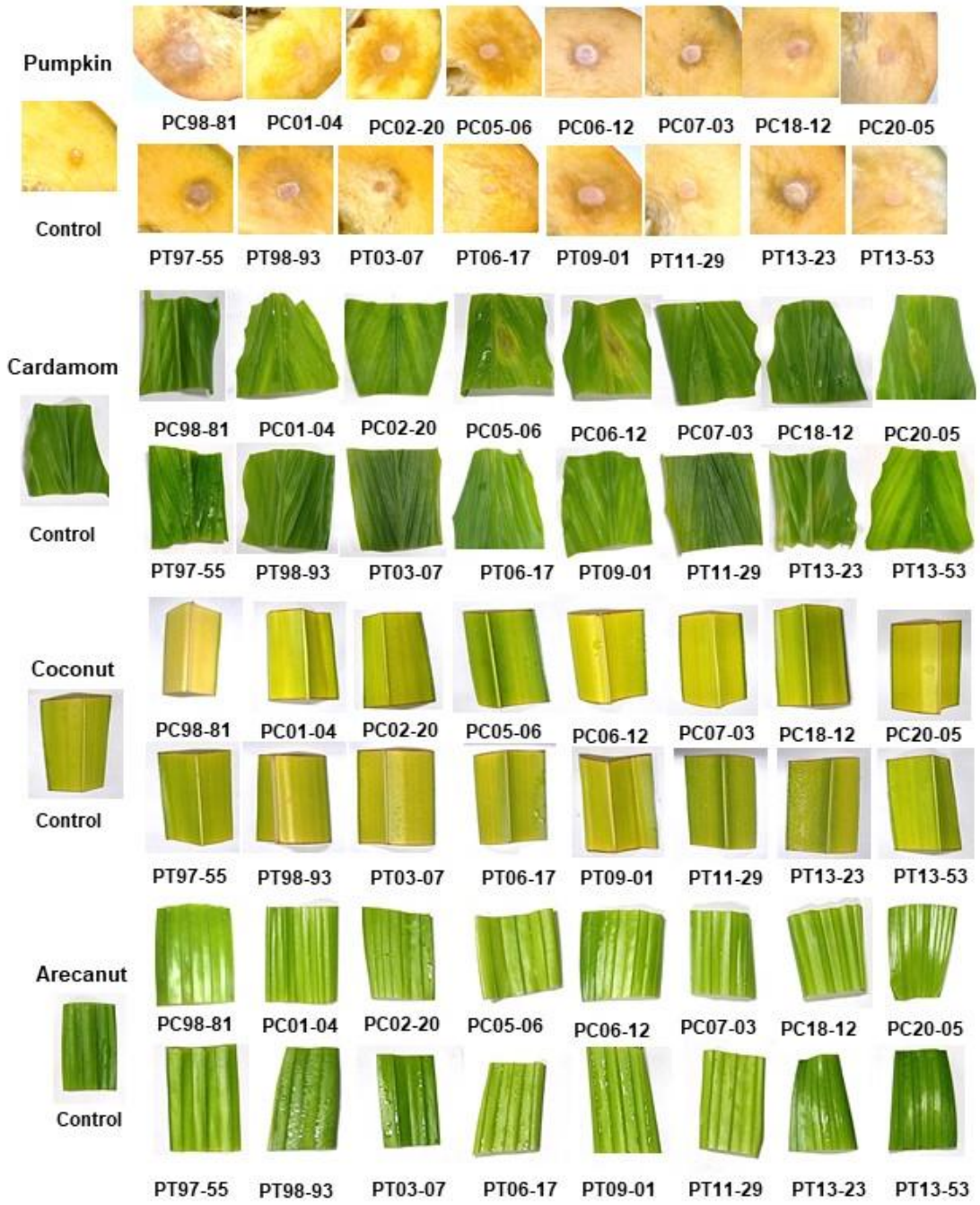


Fig. 5.9. Selected crops inoculated with black pepper *P. capsici* and *P. tropicalis* isolates showing the lesion developed on it except coconut, arecanut and vanilla

5.4. Discussion

Foot rot caused by *Phytophthora* is one of the most serious diseases that has raised concerns worldwide, as this disease is rampant if unnoticed. The disease has been reported in major black pepper growing countries like Ethiopia (Jibat & Alo, 2021), Malaysia (Farhana *et al.,* 2013) and Vietnam (Truong *et al.,* 2008). The widely studied causal organism associated with the disease includes P. *capsici, P. tropicalis* and *P. nicotianae* (Roy *et al.,* 2009). The role of *P. capsici* and *P. tropicalis* has already been described in India, and it is also important to study its coexistence. The present study accounts for the first report to explore the genetic diversity of *Phytophthora* species infecting black pepper in major black pepper growing states of India, like Karnataka, Kerala and Tamil Nadu using RAMS and REP-PCR analyses.

The variation existed among both species was conspicuous according to the present study. One of the major factors affecting the diversity analysis is the sample size. In the present study, the samples were selected based on the availability of the isolates from the National Repository of *Phytophthora*, ICAR-IISR, Kozhikode, India, hence size of the samples selected form each states varied significantly i.e., greater number of isolates were selected from Kerala and nearly half the numbers were selected from Karnataka. As far as the values were considered, diversity estimates were greater for the Kerala population, but when taking the size of the samples into account, it was observed that the Karnataka population is more diverse. The size of the samples selected from Tamil Nadu and Goa was less substantially and is inadequate to describe the genetic diversity of these populations.

Present study revealed the diversity that exist among the Indian black pepper *Phytophthora* isolates and also the analyses could clearly differentiate the two so called “sibling species” of *Phytophthora,* in fact REP-PCR analysis alone is effective in the differentiation as its UPGMA clustering patterns were similar to that of the combined analyses of RAMS and REP-PCR, however UPGMA clustering with RAMS data showed slight deviation from the aforementioned observations. Although the polymorphic loci data generated by RAMS and REP-PCR markers were almost similar, the data generated by REP-PCR were slightly greater. The

potentiality of RAMS and REP-PCR analyses and REP-PCR analysis to differentiate the two species was already been discussed moreover, the topology of the clusters obtained from the Principal Coordinate Analysis (PCoA) further supports the above observations. A number of studies have been available which used the REP-PCR analysis to study the genetic diversity of *Phytophthora* spp. In 2007, Bouws and Finckh detected the variations in the population of *P. infestans* studied using REP-PCR fingerprints. Yet another study revealed the genetic diversity of *P. palmivora* of Indonesia and Japan populations using REP-PCR primers (Masyahit *et al.,* 2019).

The isolates selected in the present study were found to be unique, and thereby the population is heterogeneous. No correlation was observed between the UPGMA clustering and the geographical location from where the isolates were collected. A similar observation was reported from Vietnam by Truong *et al*. (2010), who studied the clonal isolates of *P. capsici* infecting black pepper. Another study from India reported that the RAMS analysis of *P. colocasia* unravelled a diverse population, whereas no correlation was observed between UPGMA clustering and the place of origin of the isolate (Nath *et al.,* 2016). In contrast to the above observations, the RAMS cluster of *P. capsici* isolates infecting *Capsicum annum* in Bhutan corresponds to the place of collection of isolates (Rai *et al.,* 2020). A similar study was reported from Indonesia by Masyahit *et al*. (2019) that the REP-PCR based clustering corresponds to the geographical origin irrespective of the host or mating type.

All the Indian *Phytophthora* isolates infecting black pepper were identified as A1 (unpublished data), and the mating type of the isolates selected in the present study was also identified as A1 mating type. Nonetheless, the possibility of sexual reproduction cannot be precluded as A2 mating type was identified in South India among the *P. capsici* isolates infecting other crops like cocoa (Chowdappa & Chandramohan, 1997). Moreover, there is a high chance of interspecific hybridisation with the A2 mating type of *P. palmivora* present predominantly in Kerala and Karnataka (Chowdappa & Chandramohan, 1997). The other possibility for the heterogenous nature of black pepper *Phytophthora* isolates could be

mutations and mitotic recombination (Goodwin, 1997; Truong *et al.,* 2010; Li & Liu, 2021).

Morphological studies showed that the isolates displayed a manifold pattern of growth and modified chrysanthemum being frequent. Aragaki and Uchida (2001) differentiated the sporangial dimension between *P. capsici* and *P. tropicalis* where the former had a length-to-breadth ratio <1.8 with a broad base, and the length-to-breadth ratio of the latter was >1.8 with a tapered base. But in the present study, there was no specific pattern observed i.e., broad sporangia with round base and elongated sporangia with tapered base were observed in both species.

Phytophthora capsici is an erratic pathogen which imparts an appalling injury to its host and has a wide host range (Meitz *et al.,* 2010). *P. tropicalis* infects fruit trees (Brumat *et al.,* 2022) and ornamental plants (Hong *et al.,* 2006). The major crops which *P. capsici* infects include cucumber (Mansfeld *et al.,* 2020), tomato (Syed-Ab-Rahman *et al.,* 2019), pumpkin (Mohammad & Jose, 2018) and chilli (Majid *et al.,* 2016), and *P. tropicalis* infects crops like cucumber *in vitro* (Hong *et al.,* 2008) and tomato seedlings (Orlikowski *et al.,* 2006). The present study also analyzed the pathogenicity and virulence pattern of black pepper isolates in other crops like plantation, vegetables and spices under in vitro conditions. It is observed that apart from black pepper, the isolates could infect other crops like tomato, nutmeg, chilli, cucumber, pumpkin and cardamom. The crops like vanilla, coconut and arecanut remained uninfected. The pattern of virulence of the isolates also had no correlation with the RAMS and REP-PCR based UPGMA cluster, as there was huge variability in the pattern of symptom development both in black pepper and other crops i.e., the least aggressive isolate in one crop showed most aggressiveness in other crops.

5.5. Conclusion

RAMS and REP-PCR are among the robust and reliable techniques to study the genetic diversity. The analysis accentuated its advantage in unravelling the genetic diversity among the “sister species” of *Phytophthora* infecting black pepper. The combined analyses were capable of distinguishing the two species. When we compare the potentiality of the two techniques, the REP-PCR technique should be

emphasized as this technique was alone able to distinguish between the two species, and moreover the technique also revealed a greater number of polymorphic loci than the RAMS technique. The analyses also showed that the population is heterogeneous with unique isolates regardless of their place of origin. A variety of colony morphologies was displayed by the isolates, and all the isolates were found to be A1 mating type. The ability of the black pepper *Phytophthora* isolates in infecting crops like cucumber, pumpkin, chilli, tomato, nutmeg and cardamom (a few isolates) in vitro was also identified from the present study.

CHAPTER 6

ANALYSIS OF HAPLOTYPES IN *P. CAPSICI* AND *P. TROPICALIS* INFECTING BLACK PEPPER

Abstract

Black pepper production has been gravely struck by the wreaking havoc due to foot rot caused by *Phytophthora* species namely, *P. capsici* and *P. tropicalis*. In India, black pepper is widely cultivated in Karnataka, Kerala and Tamil Nadu. The present study included the isolates collected from these major black pepper growing regions for haplotype analysis to decipher population diversity and to characterize the colony and sporangial morphology The haplotype analysis was carried out using both mitochondrial (Cox1, Cox2, Nad1 and Nad5) and nuclear genes (β-tubulin, EF-1α, Enolase, HSP90, TigA and Ura3). Morphological characters analyzed were highly variable and the majority showed umbellate ontogeny with caducous sporangia exhibiting different shapes. Sequence analysis was performed after manually trimming and aligning the sequences using ClustalX2. DnaSP v6.12.03 was used to calculate various parameters like polymorphisms, haplotypes, haplotype and nuclear diversity, recombination events and neutrality tests. The isolates displayed a greater number of haplotypes for EF1-α and the haplotypes identified for Nad1 and Ura3 were comparatively less. PopART was used for the visual representation of the identified haplotypes. Further, the haplotypes identified from *P. capsici* infecting diverse hosts from Hawaii and some of the contiguous United States were also compared with the present study to impart more clarity. Restoration of genetic diversity after a severe bottleneck through balancing selection was revealed using the demographic analysis. The phylogenetic study also indicated a probability of population origin of the Indian black pepper *Phytophthora* population rooted back to the aforementioned US population.

6.1. Introduction

Black pepper is one of the important commercial crops of India with high economic importance. Black pepper is an important component in the Indian traditional medicine and is widely used as a condiment. Major black pepper growing states are Kerala, Karnataka and Tamil Nadu. Black pepper cultivation is severely hit by the incidence of foot rot disease caused by oomycete *P. capsici* and *P. tropicalis* (Bhai *et al.,* 2022). *P. capsici,* being a polycyclic pathogen, causes a devastating loss of accounts following the infection (Rini & Remya, 2020). *Phytophthora* infection in black pepper results in spike shedding, reduction in bush size, root loss, rotting of shoots, foot rot, dark brown lesions on leaves, foliar yellowing and defoliation (Sarma & Anandaraj, 1997).

Sexual reproduction of *Phytophthora* renders additional benefit to the pathogen; these are survival structures called oospores characterized by the presence of amphigynous antheridia with a thick-walled globose oogonia that persists in soil for years (Gobena *et al.,* 2012; Manohara, 2007), and it aids the genetic variation among the population. Various approaches to study the genetic variation includes RAPD (Sun *et al.,* 2008), haplotype identification using mitochondrial and nuclear genes (Quesada-Ocampo *et al.,* 2011a), SNP and AFLP (Gobena *et al.,* 2012), simple sequence repeat (Wang *et al.,* 2010; Afaf *et al.,* 2009), PCR-RFLP (Ibrahim *et al.,* 2012) and comparative genome analysis (Lee *et al.,* 2021).

Haplotypes are the combination of alleles at a particular locus on a chromosome, that inherits together (Garg, 2021) or it can be defined as a set of SNPs with strong linkage disequilibrium (Bhat *et al.,* 2021); the difference in the phenotypic trait is directly linked to the genetic variation that exist for that particular trait (Hughes *et al.,* 2008). Sexual reproduction gives rise to heterozygous progeny with differences in haplotype frequency (Carlson *et al.,* 2017). Steps involved in haplotype analysis include the determination of haplotype blocks, haplotyping and finding the haplotype association (Liang, 2013). Analysis of haplotypes helps to unravel the nexus of genetic variations that exist within a genome, which occurred as a result of complex evolutionary forces. An insight into the diversity and distribution of the

pathogen population in a given area is critical for its management (Kashyap *et al.,* 2022).

Both mitochondrial and nuclear genes contribute significantly in understanding the haplotype. The mitochondrial DNA (mtDNA) is maternally inherited circular DNA with mutation rates greater than tenfold due to the absence of an optimal pathway for DNA repair (Bi *et al.,* 2023), it lacks protective histones, and it has relatively low occurrences of recombination events (Vadakedath *et al.,* 2023). Studying the nuclear genes besides mtDNA would enhance the knowledge on population structure (Garagnani *et al.,* 2014). The present study aimed to study the colony and sporangial morphology and also to identify the haplotypes of *P. capsici* and *P. tropicalis*, based on mitochondrial and nuclear genes.

6.2. Materials and Methods

6.2.1. Isolate selection and maintenance

P. capsici and *P. tropicalis* isolates infecting black pepper collected during the period of 1997-2020 from different states were obtained from the National Repository of *Phytophthora* at ICAR-Indian Institute of Spices Research, Kozhikode and used in the study. Species identification of the isolates was done using PCR with ITS (Bhai *et al.,* 2022) and Ypt1 gene-based species-specific primers (Jeevalatha *et al.,* 2021). A total of 24 isolates from Kerala, Karnataka, Tamil Nadu and Goa were selected for the study (Table 6.1; Fig. 6.1). Pure cultures of these isolates were maintained in a Petri plate containing carrot agar medium (Patil *et al.,* 2022). For long-term storage, the agar plugs were maintained in sterile distilled water.

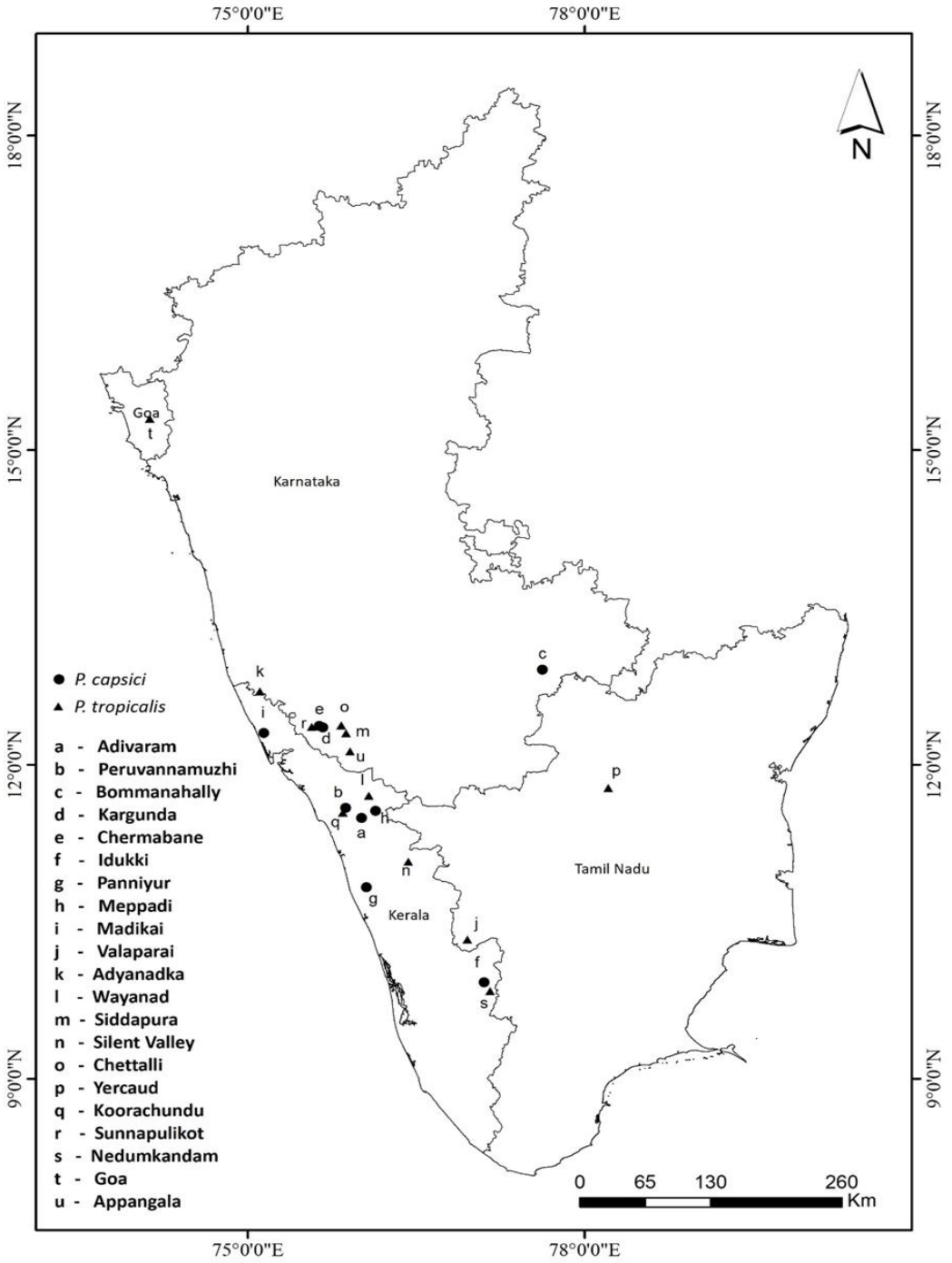


Fig. 6.1. Geographical distribution of the black pepper *Phytophthora* isolates used in the study

Table 6.1. Source and year of collection of selected *P. capsici* and *P. tropicalis* isolates infecting black pepper

Sl. No	Isolate	Species	Mating type	Place of collection	Year of collection
1	01-04	*P. capsici*	A1	Adivaram, Kozhikode	2001
2	05-06	*P. capsici*	A1	Peruvannamuzhi, Kozhikode	2005
3	10-02	*P. capsici*	A1	Bommanahally, Bangalore	2010
4	11-13	*P. capsici*	A1	Kargunda, Kodagu	2011
5	11-16	*P. capsici*	A1	Cherambane, Kodagu	2011
6	18-12	*P. capsici*	A1	Idukki	2018
7	20-05	*P. capsici*	A1	Panniyur, Kannur	2020
8	13-17	*P. capsici*	A1	Meppadi, Wayanad	2013
9	13-42	*P. capsici*	A1	Madikai, Kasargod	2013
10	97-55	*P. tropicalis*	A1	Valparai, Coimbatore	1997
11	98-93	*P. tropicalis*	A1	Adyanadka, Dakshina Kannada	1998
12	99-136	*P. tropicalis*	A1	Wayanad	1999
13	00-38	*P. tropicalis*	A1	Sidapur, Kodagu	2000
14	01-20	*P. tropicalis*	A1	Silent Valley, Palakkad	2001
15	03-07	*P. tropicalis*	A1	Adivaram, Kozhikode	2003
16	05-17	*P. tropicalis*	A1	Chettalli, Kodagu	2005
17	06-17	*P. tropicalis*	A1	Rasi Estate, Yercaud, Salem	2006
18	08-07	*P. tropicalis*	A1	Koorachundu, Kozhikode	2008
19	09-01	*P. tropicalis*	A1	Peruvannamuzhi, Kozhikode	2009
20	11-20	*P. tropicalis*	A1	Sunnapulikot, Kodagu	2011
21	11-29	*P. tropicalis*	A1	Wayanad	2011
22	13-23	*P. tropicalis*	A1	Nedumkandam, Idukki	2013
23	13-53	*P. tropicalis*	A1	Goa	2013
24	16-06	*P. tropicalis*	A1	Appangala, Kodagu	2016

Morphological characterization

Phytophthora isolates were grown in carrot agar medium, and the growth patterns were recorded on the fifth day after inoculation. To study the sporangial characters, 5mm discs were cut out from a 3-day-old culture and placed in a Petri plate containing sterile water under continuous light for 48 hours at 25±1^{o}C for sporangia production. The sporulated discs were observed under light microscope, Leica DM 5000 B (Leica Mikrosystems Vertrich GmbH, Germany), to study the dimensions. A

total of 50 readings were recorded for each isolate to study the morphology of sporangia. Heat map analysis for various morphological characters like mean l/b ratio, pedicel length, chlamydospore formation, ontogeny, papilla formation, colony morphology and sporangial shape was performed using R Studio R version 4.2.1 (R Core Team 2022).

6.2.2. DNA extraction

Agar plugs from a 3-day-old culture were inoculated into Ribeiro's broth (Erwin & Ribeiro, 1996) for the mass growth of mycelia and were harvested on the 4th day and blotted on sterile filter paper to remove the dampness. DNA from *Phytophthora* mycelia was extracted by the phenol-chloroform method according to Sheji *et al.* (2009) with slight modifications (Jeevalatha *et al.,* 2021).

6.2.3. PCR amplification and DNA sequencing

The regions of mitochondrial (Cox1, Cox2, Nad1 and Nad5) and nuclear genes (β-tubulin, EF1α, Enolase, HSP90, TigA and Ura3) were amplified by PCR assay according to Quesada-Ocampo *et al*. (2011b, Table 6.2). PCR was carried out with a total volume of 20 µl composed of 1 µl of 150ng/ µl template DNA, 2 µl Taq Buffer A (GeNei^{TM}), 0.5 µl dNTP Mix 2mM (Thermo Scientific), 0.5 µl each of forward and reverse primer, 0.3 µl Taq DNA Polymerase (GeNei^{TM}), 15.2 µl nuclease free water (Himedia). PCR was performed in ProFlex PCR system (Applied biosystems) by following the cycling conditions: 5 min of denaturation at 95^{o}C, followed by 35 cycles of 1 min at 94^{o}C, annealing at 56^{o}C for all the genes except HSP90 which is 61^{o}C for 1 min, extension at 72^{o}C for 1 min and final extension of 72^{o}C for 10 min. The PCR products were run in 1% agarose gel stained with ethidium bromide, and were purified using GenElute^{TM} Gel Extraction Kit (Sigma-Aldrich, Munich, Germany) by following the manufacturer's instructions. The purified PCR products were sequenced using the Sanger sequencing method.

Table 6.2. List of primers used for the analysis of haplotypes for the selected mitochondrial and nuclear genes (Quesada-Ocampo *et al.,* 2011b)

Gene	Primer	Size of sequenced region	Size of analyzed region
Mitochondrial			
Cox1	Cox1-F 5′GGTGCACCTGATATGGCTTT3′ Cox1-R 5′ACAGGATCACCTCCACCTGA3′	425 bp	325 bp
Cox2	Cox2-F 5′CCAGCAACTCCTGTAATGGAA3′ Cox2-R 5′TTGATTTAAACGGCCAGGAC3′	540 bp	365 bp
Nad1	Nad1-F 5′CAAAGAAGAAGAGGACCTAATGTTG3′ Nad2-R 5′TAATGCAAAACCCATTGCAG3′	579 bp	356 bp
Nad5	Nad5-F 5′GCTATGGAAGGTCCTACACCA3′ Nad5-R 5′GCATGGATTACTGCACCTGA3′	341 bp	245 bp
Nuclear			
β-tubulin	BTub-F 5′GGTCAGTGCGGTAACCAGAT3′ BTub-R 5′GTACAGGGCCTCGTTATCCA3′	597 bp	484 bp
EF-1α	EF1A-F 5′GACATTGCCCTGTGGAAGTT3′ EF1A-R 5′CAGGCTTGATGACACCAGTC3′	568 bp	450 bp
Enolase	Enolase-F 5′CGTGAAGAACGTGAACGAGA3′ Enolase-R 5′CCGAGATCTTCTCCGACTCC3′	647 bp	341 bp
HSP90	HSP90-F 5′GCCGATCTCATCAACAACCT3′ HSP90-R 5′CTTCTGCGAGTTCAGGTGGT3′	542 bp	465 bp
TigA	TigA-F 5′TCAACACTGCCAAAATTCCA3′ TigA-R 5′CAGCGTCAGAGGAGACCTTC3′	516 bp	420 bp
Ura3	Ura3-F 5′GGCTTTCGACCAGCTGAAT3′ Ura3-R 5′AGCGTGAAGTCACCGAACTT3′	570 bp	432 bp

6.2.4. Sequence Analysis

Sequences were manually edited using BioEdit version 7.2.5 (Hall, 1999), and multiple sequence alignment was performed using ClustalX2 (Larkin *et al.,* 2007). The sequence data was saved as NEXUS file format, which is to be used as an input for other software. Gene genealogy was estimated for *Phytophthora* isolates from India infecting black pepper by implementing statistical parsimony. PopART was used to analyze population genetics by haplotype network (Clement *et al.,* 2002). DnaSP v6.12.03 was used to study polymorphisms, haplotype, recombination events and neutrality tests of the mitochondrial and nuclear genes (Rozas *et al.,* 2017). The haplotypes identified from the black pepper isolates were compared with the isolates from diverse host Hawaii and some of the contiguous United States such as Arizona, California, Delaware, Florida, Georgia, Kentucky, Louisiana, Massachusetts, Michigan, New Jersey, New Mexico, New York, North Carolina, Ohio, Oklahoma, South Carolina, Tennessee, Texas (Quesada-Ocampo *et al.,* 2011b) for each gene to identify the unique haplotypes. The GenBank accessions HQ388837 – HQ389193 were retrieved from NCBI.

6.2.5. Phylogenetic analysis

The resulting nucleotide sequences after editing and trimming were used for the phylogenetic analysis. The sequences were aligned using ClustalX2 (Larkin *et al.,* 2007) and saved in FASTA format. The phylogenetic tree was constructed for concatenated mitochondrial and nuclear genes separately and also by combining both. Tree was also constructed for Indian black pepper isolates along with the isolates from diverse hosts of Hawaii and some of the contiguous United States (Quesada-Ocampo *et al.,* 2011b). Molecular Evolutionary Genetics Analysis (MEGA) version 11 (Tamura *et al.,* 2021) was used for the construction of phylogenetic tree using Maximum Likelihood method with Kimura 2-parameter model, and it was run for 1000 bootstrap replications.

6.3. Results

6.3.1. Culture characterization and morphology

A total of 24 isolates, 12 each of *P. capsici* and *P. tropicalis* collected over a period of time from four different states were used in this study. Various morphological

characters like mean l/b ratio of sporangia, pedicel length, chlamydospore formation, ontogeny, papilla formation, colony morphology and sporangial shape were studied (Table 6.3). Heat map was constructed to depict the morphological characteristics of the isolates.

Based on similar distributions along the column of the heatmap, the morphological characters are broadly grouped into two, where sporangial shape and colony morphology are grouped as one cluster and other characters such as ontogeny, chlamydospore formation, pedicel length, mean length/breadth and length/breadth range were grouped into another. Based on vector similarities, the isolates were clustered into two and there was no clear distinction between the two species as these were randomly distributed (Fig. 6.2).

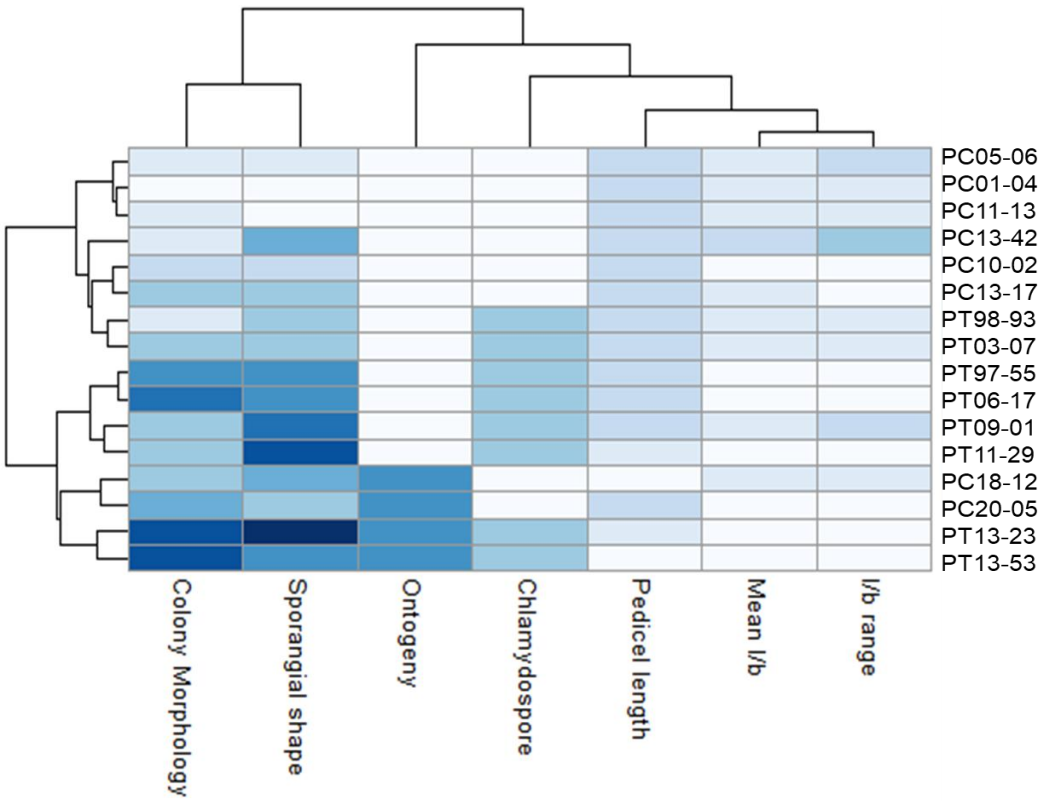


Fig. 6.2. Heatmap showing the morphological characters

Table 6.3. Morphological characters of selected *Phytophthora* isolates

Sl. No.	Isolate	Morphology	Ontogeny	Caducity	Mean l/b	l/b range	*Pedicel	*Sporangia	Chlamydospore
1	PC01-04	Stellate with concentric rings	Umbellate	Caducous	2.65	1.8-3.5	*L	Naviculate	*A
2	PC05-06	Modified chrysanthemum	Umbellate	Caducous	2.5	1.8-4.0	L	Elongated	A
3	PC10-02	Cottony with concentric rings	Umbellate	Caducous	1.44	1.2-1.73	L	Globose	A
4	PC11-13	Modified chrysanthemum	Umbellate	Caducous	2.1	1.6-2.7	L	Naviculate	A
5	PC13-17	Modified stellate	Umbellate	Caducous	2.06	1.0-2.7	L	Elliptical	A
6	PC13-42	Modified chrysanthemum	Umbellate	Caducous	3.2	2.5-5	L	Pyriform	A
7	PC18-12	Modified stellate	Sympodial	Caducous	2.58	2.12-3.32	*S	Pyriform	A
8	PC20-05	Stellate	Sympodial	Caducous	1.85	1.43-2.31	L	Elliptical	A
9	PT97-55	No pattern	Umbellate	Caducous	1.6	1.2-1.9	L	Limoniform	*P
10	PT98-93	Modified chrysanthemum	Umbellate	Caducous	2	1.7-2.4	L	Elliptical	P
11	PT03-07	Modified stellate	Umbellate	Caducous	2.0	1.5-4.0	L	Elliptical	P
12	PT06-17	Modified petaloid	Umbellate	Caducous	1.68	1.2-2.5	L	Limoniform	P
13	PT09-01	Modified stellate	Umbellate	Caducous	2.2	2.0-4.5	L	Ovoid-obpyriform	P
14	PT11-29	Modified stellate	Umbellate	Caducous	1.42	1.2 -1.71	*M	Ovoid	P
15	PT13-23	Stellate with cottony mycelia	Sympodial	Caducous	1.37	1.17-1.61	M	Obpyriform	P
16	PT13-53	Stellate with cottony mycelia	Sympodial	Caducous	1.47	0.15-1.85	S	Limoniform	P

*Morphology- Colony morphology, *Pedicel- Length of pedicel, *Shape- Sporangial shape, *A- Absent, *P- Present, *L- Large, *S- Small, *M- Medium

6.3.2. Haplotype analysis

Mitochondrial and nuclear genes were sequenced for all the isolates, and the sequences were submitted to NCBI (Appendix). A total of 96 polymorphic regions were identified for the mitochondrial and nuclear genes, out of which 90 were parsimony-informative sites. 24 were synonymous nucleotide changes, and 72 were replacement changes. Sixty-seven of which were transitions and 29 were transversions (Table 6.4). The parsimony informative site was greater for Nad5 followed by Nad1 in the case of mitochondrial gene, and lowest for Cox2 region of the gene. Whereas for nuclear genes, TigA region has a greater parsimony informative site followed by Ura3 and β-tubulin and lesser for Enolase. Unlike nuclear genes, synonymous changes were not identified in mitochondrial genes, and all the nucleotide changes in β-tubulin and HSP90 regions were synonymous. No synonymous changes were identified in TigA region as all are of replacement changes. All the nucleotide changes identified for mitochondrial genes were replacement changes. In case of nuclear genes, TigA has a greater number of replacement changes followed by Ura3 and lesser replacement changes were identified for Enolase. Among the nucleotide changes, the majority of the changes were transitions except for EF1-α. In case of mitochondrial genes, the replacement changes were four transitions and one transversion in the Cox1 region, in the Cox2 region, all the replacements were transitions. In Nad1 region, four transitions and three transversions and for Nad5, eight transitions and two transversions were identified. In nuclear genes, transition and transversion changes were more at TigA region and less at Enolase and HSP90.

Haplotype analysis identified a total of 30 haplotypes from the mitochondrial and nuclear genes. A greater number of haplotypes (five) were identified for the EF1-α gene (Table 6.5), three haplotypes each for Cox1, Cox2, Nad5, β-tubulin, enolase, HSP90 and TigA gene and two haplotypes each for Nad1 and Ura3 were identified. The haplotype diversity and nucleotide diversity range from 0.489 to 0.757 and 0.0027 to 0.020, respectively. Haplotype diversity was highest for EF1-α and uniform for Cox1, Cox2, Nad5 and β-tubulin. Minimum number of recombination

events was absent except for EF1-α. Neutrality test computed by Tajima's D, Fu and Li's F and D values were positive.

Polymorphic loci were identified for both mitochondrial and nuclear genes. In case of mitochondrial genes, greater number of polymorphisms were identified at Nad5 region with three haplotypes accounting for haplotype diversity of 0.685. Nad1 has seven polymorphisms with 2 haplotypes accounting for haplotype diversity of 0.489. Cox1 and Cox2 respectively have 5 and 3 polymorphisms, with 3 haplotypes each having haplotype diversity similar to Nad5. Watterson's theta estimator per gene from sequence and average number of nucleotide differences were also greater for Nad5. For nuclear genes, greater polymorphism was identified for TigA followed by Ura3 and β-tubulin same trend follows for Watterson's theta estimator per gene from sequence and average number of nucleotide differences, but greater haplotypes were identified for EF1-α region with nine polymorphisms, with greater haplotype diversity of 0.757. The least number of polymorphisms was identified for Enolase, with three haplotypes having haplotype diversity of 0.67. Haplotype identified for the Nad1 region of the mitochondrial gene and the Ura3 region of the nuclear gene was the least.

Table 6.4. Summary of polymorphism for the target mitochondrial and nuclear genes in *P. capsici* and *P. tropicalis*

Target DNA	Parsimony informative site	Synonymous changes	Replacement changes	Transition	Transversion
Mitochondrial					
Cox1	5	0	5	4	1
Cox2	3	0	3	3	0
Nad1	7	0	7	4	3
Nad5	10	0	10	8	2
Nuclear					
β-tubulin	13	13	0	10	3
EF1-α	9	1	8	3	6
Enolase	4	3	1	3	1
HSP90	5	5	0	3	2
TigA	18	0	24	17	7
Ura3	16	2	14	12	4
Total	90	24	72	67	29

Table 6.5. Diversity, recombination and neutrality estimates for the mitochondrial (Cox1, Cox2, Nad1 and Nad5) and nuclear (B-tubulin, EF1 α, TigA, Ura3) genes in *P. capsici and P. tropicalis*

	Diversity estimates					Recombination estimates			Neutrality test	
Target DNA	**S**	**h**	**Hd**	**π**	**Θw**	**k**	**R**	**RM**	**Tajima's D**	**Fu and Li's D**	**Fu and Li's F**
Mitochondrial											
Cox1	5	3	0.685	0.00663	1.339	2.446	1.9	0	2.39529	1.16632	1.75945
Cox2	3	3	0.685	0.00273	0.803	1.370	13.8	0	1.77272	0.97946	1.38696
Nad1	7	2	0.489	0.00670	1.875	3.424	0.001	0	2.58339	1.28730	1.93382
Nad5	10	3	0.685	0.01545	2.678	4.696	2.4	0	2.51394	1.40764	2.02080
Nuclear											
β-tubulin	13	3	0.685	0.01243	3.481	6.163	0.2	0	2.67252	1.48823	2.15006
EF1-α	9	5	0.757	0.00867	2.410	4.431	2.2	2	2.74857	1.37323	2.06984
Enolase	4	3	0.670	0.00411	1.071	1.975	3.1	0	2.30679	1.08443	1.65539
HSP90	5	3	0.583	0.00514	1.339	2.475	0.5	0	2.45803	1.16632	1.78030
TigA	24	3	0.540	0.02099	6.427	9.467	0.001	0	1.75362	0.17310	0.77055
Ura3	16	2	0.489	0.01575	4.285	7.826	0.001	0	2.94317	1.54623	2.29917

*S = number of polymorphic sites, h = number of haplotypes, Hd = haplotype diversity, π = nucleotide diversity, Θw = theta (per site) form S, k = average number of pairwise nucleotide difference, R = estimate of recombination parameter, Rm = minimum number of recombination events.

Table 6.6. Haplotypes identified for the target mitochondrial and nuclear genes of *P. capsici* and *P. tropicalis* isolates infecting black pepper

Gene	Haplotypes	Isolates
Mitochondrial		
Cox1	Hap_1	PC01-04, PC05-06, PC10-02, PC11-13, PC11-16, PC13-17, PC13-42, PC18-12, PC20-05
	Hap_2	PT99-136, PT97-55, PT06-17, PT09-01, PT11-20, PT11-29
	Hap_3	PT05-17, PT16-06, PT98-93, PT00-38, PT01-20, PT03-07, PT08-07, PT13-23, PT13-53
Cox2	Hap_1	PC01-04, PC05-06, PC10-02, PC11-13, PC11-16, PC13-17, PC18-12, PC20-05, PT08-07
	Hap_2	PT99-136, PT97-55, PT06-17, PT09-01, PT11-20, PT11-29
	Hap_3	PT05-17, PT16-06, PC13-42, PT98-93, PT00-38, PT01-20, PT03-07, PT13-23, PT13-53
Nad1	Hap_1	PC01-04, PC05-06, PC10-02, PC11-13, PC11-16, PC13-17, PC13-42, PC18-12, PC20-05
	Hap_2	PT99-136, PT05-17, PT16-06, PT97-55, PT98-93, PT00-38, PT01-20, PT03-07, PT06-17, PT08-07, PT09-01, PT11-20, PT11-29, PT13-23, PT13-53
Nad5	Hap_1	PC01-04, PC05-06, PC10-02, PC11-13, PC11-16, PC13-17, PC13-42, PC18-12, PC20-05
	Hap_2	PT99-136, PT97-55, PT06-17, PT09-01, PT11-20, PT11-29
	Hap_3	PT05-17, PT16-06, PT98-93, PT00-38, PT01-20, PT03-07, PT08-07, PT13-23, PT13-53
Nuclear genes		
β-tubulin	Hap_1	PC01-04, PC05-06, PC10-02, PC11-13, PC11-16, PC13-17, PC13-42, PC18-12, PC20-05
	Hap_2	PT97-55, PT06-17, PT09-01, PT11-20, PT11-29, PT99-136
	Hap_3	PT98-93, PT00-38, PT01-20, PT03-07, PT08-07, PT13-23, PT13-53, PT05-17, PT16-06

EF-1α	Hap_1	PC01-04, PC05-06, PC10-02, PC11-13, PC11-16, PC13-17, PC13-42, PC18-12, PC20-05
	Hap_2	PT99-136, PT97-55, PT06-17, PT09-01, PT11-20, PT11-29
	Hap_3	PT05-17, PT16-06, PT98-93, PT00-38, PT03-07, PT13-23
	Hap_4	PT01-20 PT08-07
	Hap_5	PT13-53
Enolase	Hap_1	PC01-04, PC05-06, PC10-02, PC11-13, PC11-16, PC13-17, PC13-42, PC18-12, PC20-05
	Hap_2	PT99-136, PT97-55, PT06-17, PT09-01, PT11-29
	Hap_3	PT05-17, PT16-06, PT98-93, PT00-38, PT01-20, PT03-07, PT08-07, PT11-20, PT13-23, PT13-53
HSP90	Hap_1	PC01-04, PC05-06, PC10-02, PC11-13, PC11-16, PC13-17, PC13-42, PC18-12, PC20-05
	Hap_2	PT99-136, PT05-17, PT16-06, PT00-38, PT01-20, PT03-07, PT06-17, PT08-07, PT09-01, PT11-20, PT11-29, PT13-23, PT13-53
	Hap_3	PT97-55, PT98-93
TigA	Hap_1	PC01-04, PC05-06, PC10-02, PC11-13, PC11-16, PC13-17, PC13-42, PC18-12, PC20-05
	Hap_2	PT99-136, PT05-17, PT16-06, PT98-93, PT00-38, PT01-20, PT03-07, PT06-17, PT08-07, PT09-01, PT11-20, PT11-29, PT13-23, PT13-53
	Hap_3	PT97-55
Ura3	Hap_1	PC01-04, PC05-06, PC10-02, PC11-13, PC11-16, PC13-17, PC13-42, PC18-12, PC20-05
	Hap_2	PT99-136, PT05-17, PT16-06, PT97-55, PT98-93, PT00-38, PT01-20, PT03-07, PT06-17, PT08-07, PT09-01, PT11-20, PT11-29, PT13-23, PT13-53

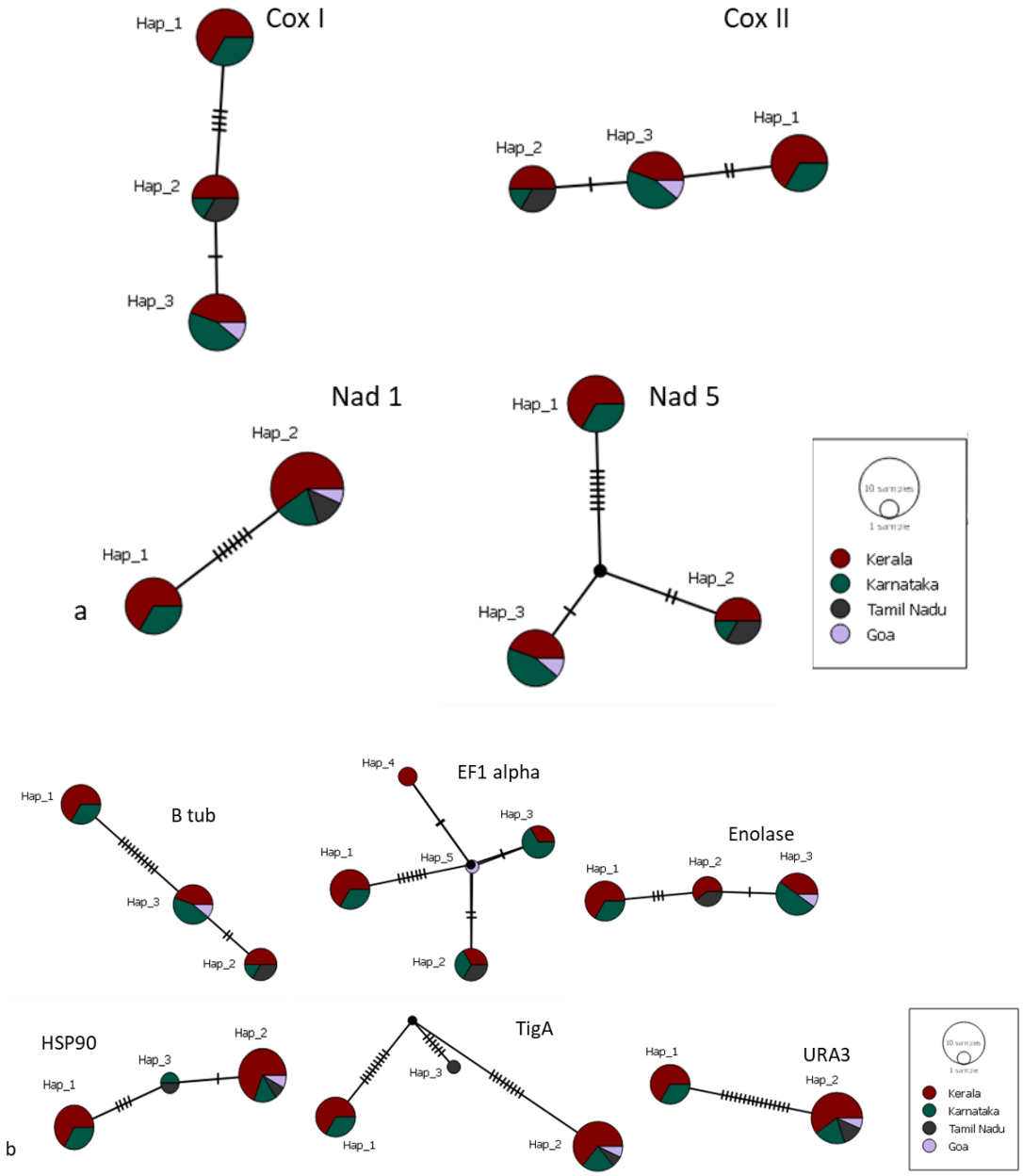


Fig. 6.3. Haplotype network a) Mitochondrial genes; b) Nuclear genes

The Haplotype network was plotted using PopART (Population Analysis using Reticulate Trees) for the mitochondrial and nuclear genes individually, and also after combining both. In mitochondrial genes three haplotypes each for Cox I, Cox II and Nad 5 were identified whereas, in Nad 1 the number of haplotypes identified was 2 (Fig. 6.3a). In nuclear genes, a greater number of haplotypes were identified for EF1

alpha (Fig. 6.3b). The list of isolates with different haplotypes for each of the gene was listed in Table 6.6. The geographical distribution of the haplotypes found in each gene of black pepper isolates is depicted using a facet chart in Fig. 6.4.

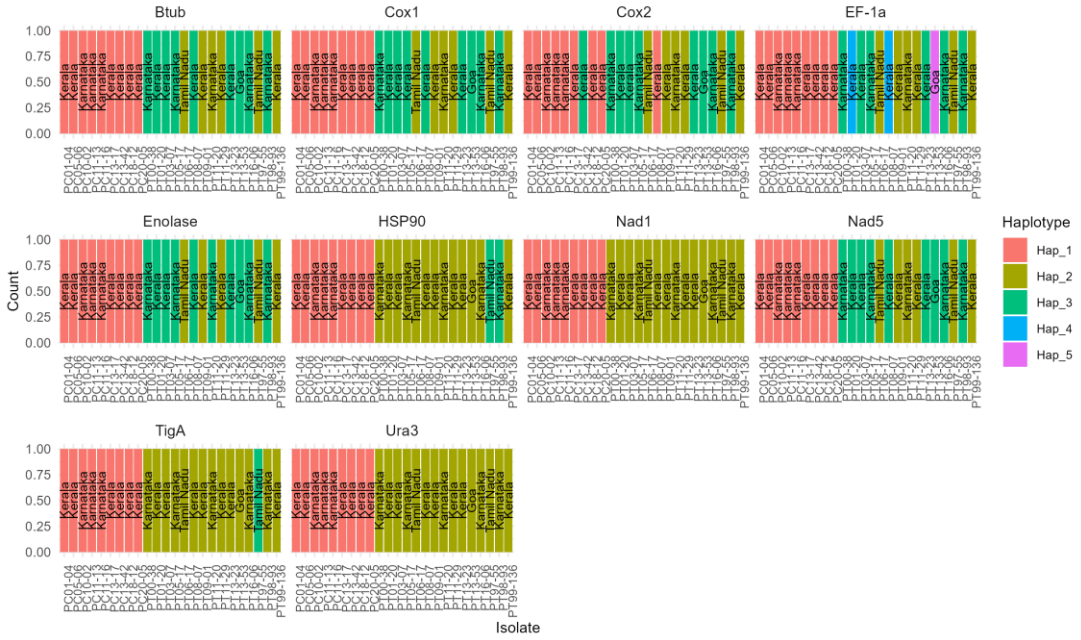
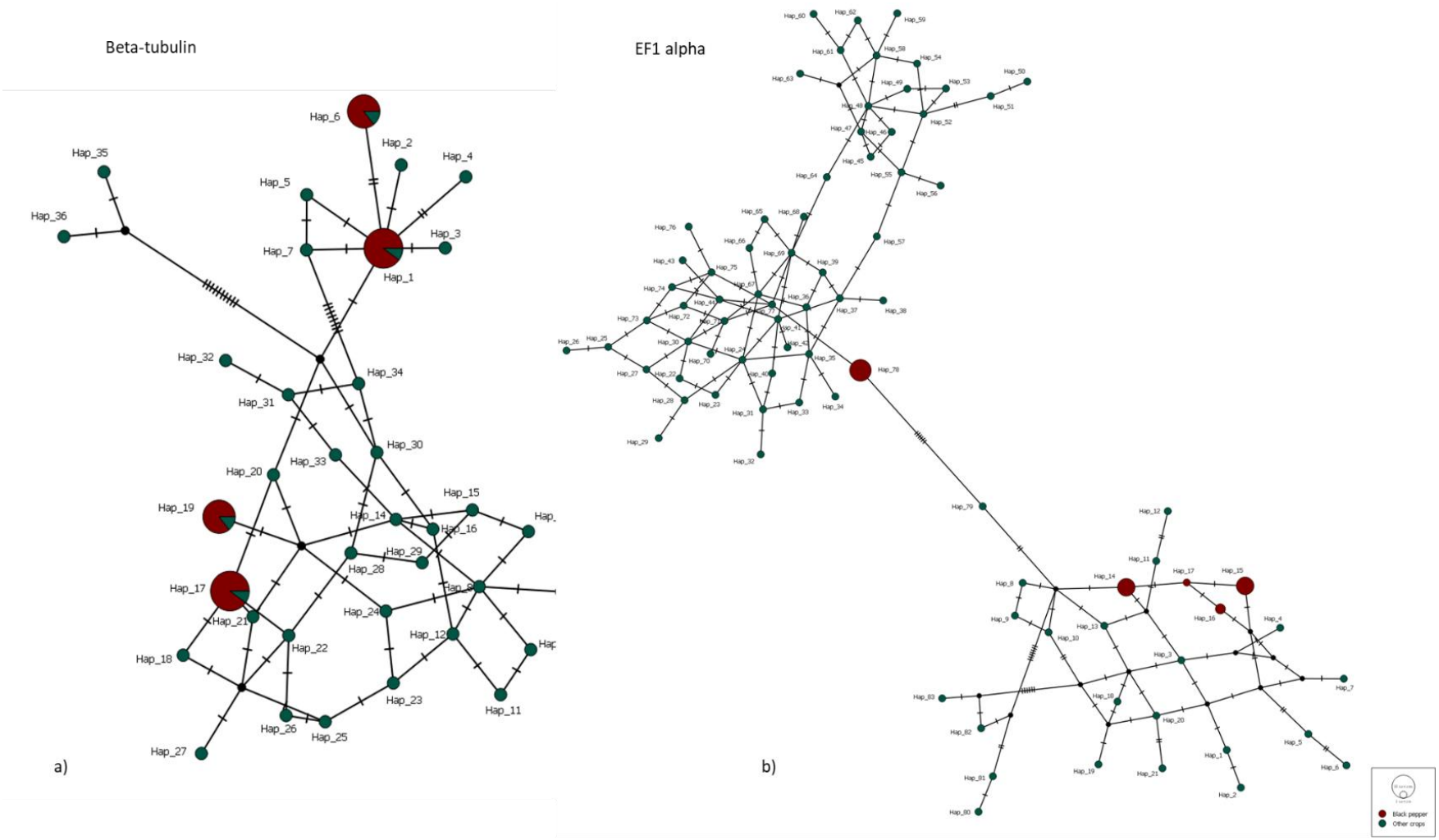


Fig. 6.4. Facet plot showing the distribution of various haplotypes identified in each of the selected genes


Beta-tubulin
Hap_6
Hap_2
Hap_4
Hap_35
Hap_5
Hap_36
Hap_3
Hap_7
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Hap_34
Hap_31
Hap_30
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Hap_24
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Hap_22
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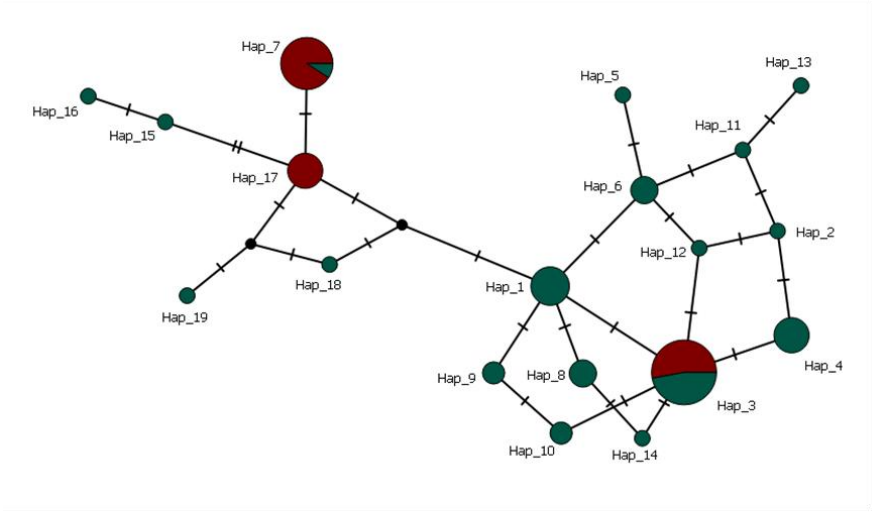
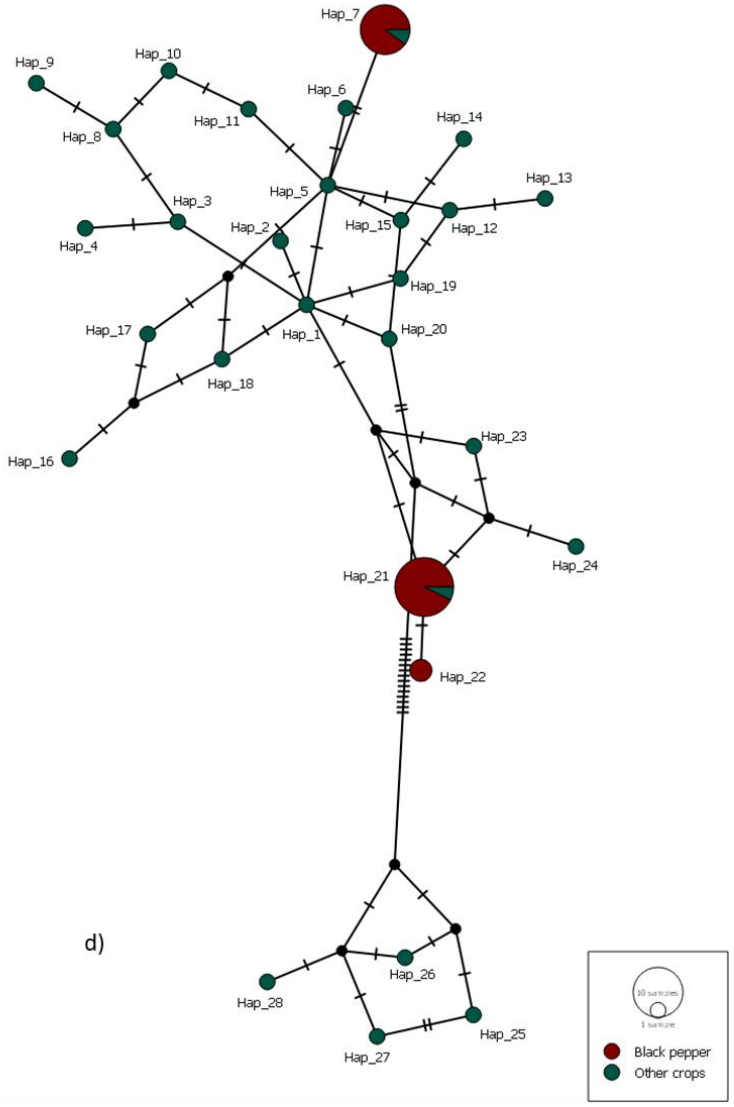
Hap_25
Hap_27
a)
EF1 alpha
b)
Black pepper
Other crops

Enolase

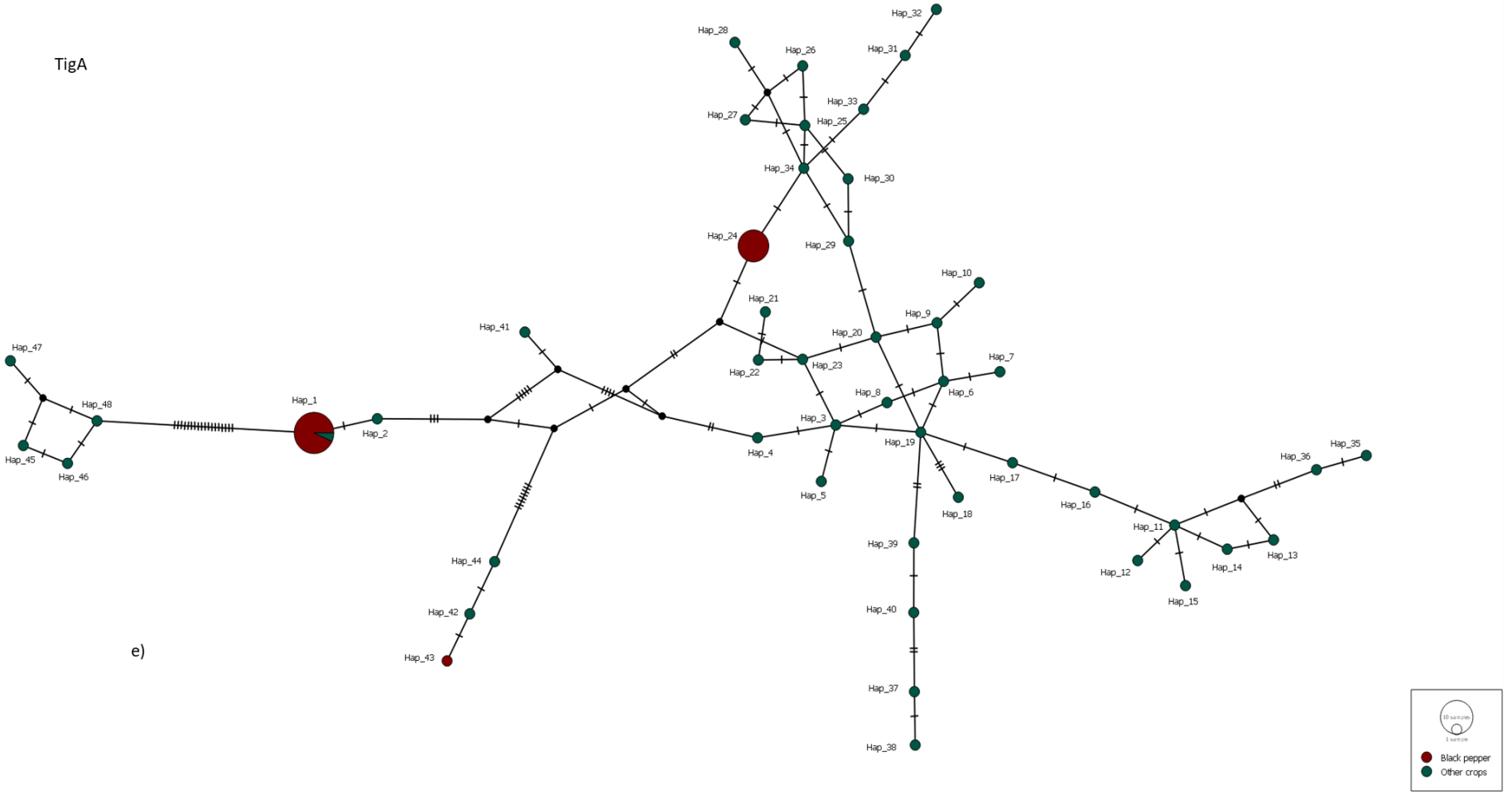


c)

HSP90



d)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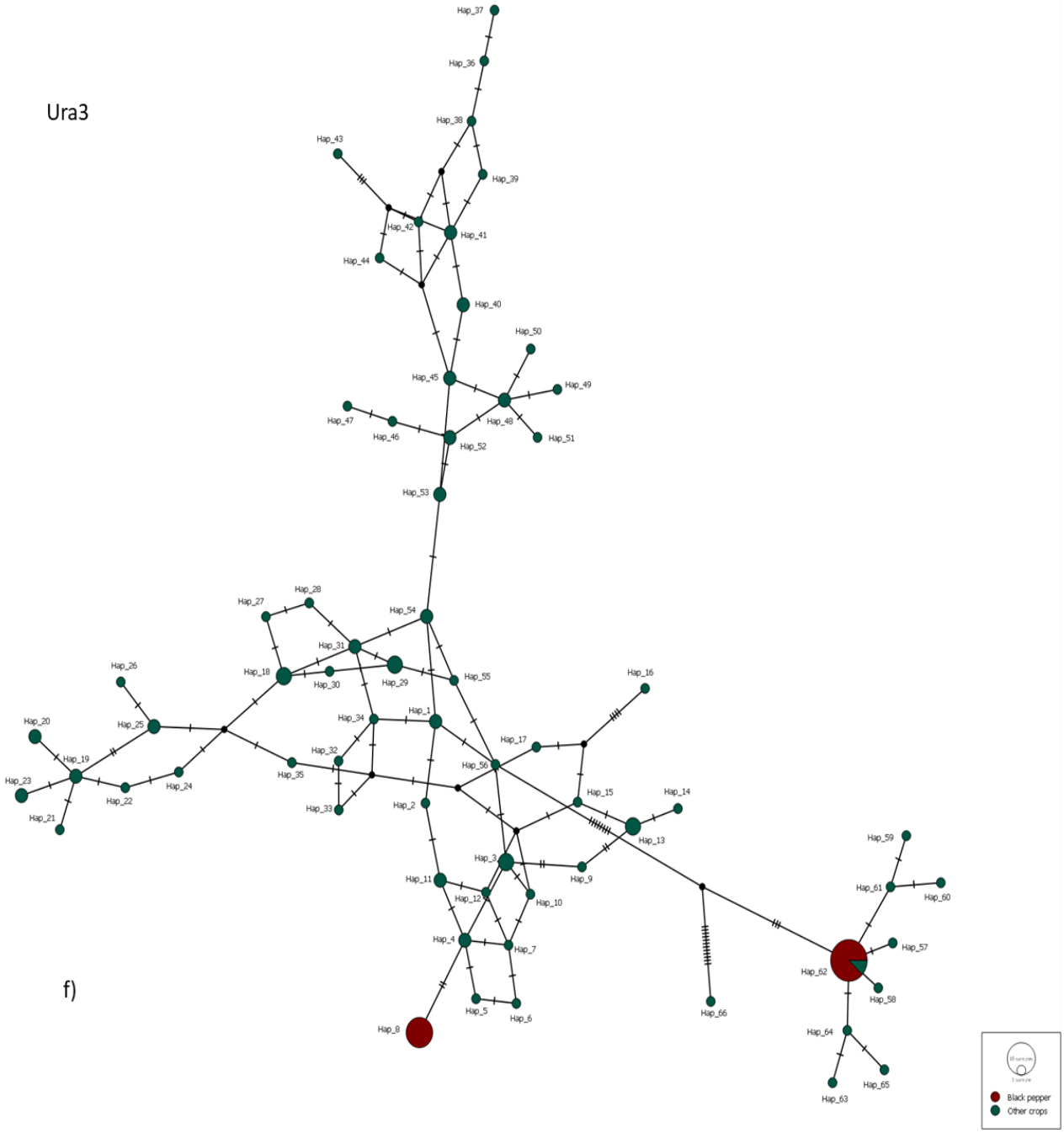
Hap_48
Black pepper
Other crops

Ura3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Hap_30
Hap_20
Hap_34
Hap_1
Hap_25
Hap_17
Hap_32
Hap_19
Hap_56
Hap_23
Hap_35
Hap_15
Hap_14
Hap_24
Hap_33
Hap_2
Hap_22
Hap_13
Hap_21
Hap_59
Hap_3
Hap_9
Hap_11
Hap_61
Hap_60
Hap_12
Hap_10
Hap_4
Hap_7
Hap_57
Hap_62
Hap_58
Hap_5
Hap_66
Hap_6
Hap_8
Hap_64
Hap_65
Hap_63
Black pepper
Other crops

f)

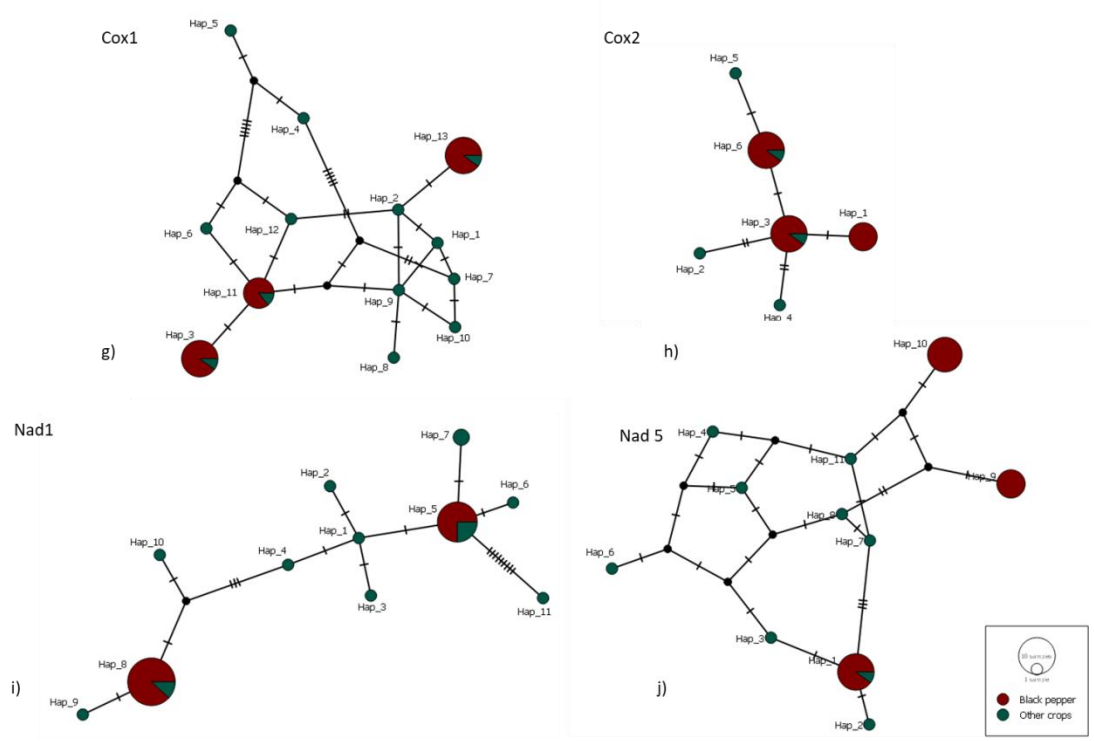


Fig. 6.5. Gene genealogy analysis of *Phytophthora* isolates from Hawaii and some of the contiguous United States (Quesada-Ocampo *et al.,* 2011b) and black pepper isolates from India. a- f) Nuclear genes; g- j) Mitochondrial genes. The size of the circle corresponds to the size of the population and the small lines on the line connecting two haplotypes represent the number of nucleotide changes

Maximum Spanning Network was also plotted with the *P. capsici* isolates of Hawaii and some of the contiguous United States (Quesada-Ocampo *et al.,* 2011b) and *P. capsici* and *P. tropicalis* isolates of India which were isolated from black pepper to identify the presence of unique haplotypes (Fig. 6.5). It was observed that the nuclear genes such as EF1alpha, enolase, HSP90, TigA and Ura3 and mitochondrial genes such as Cox2 and Nad5 incorporates unique haplotypes and it was greater for EF1 alpha. The unique haplotypes identified in nuclear genes includes Hap_2, 3 and 4 in EF1 alpha, Hap_2 in enolase, Hap_3 in HSP90, Hap_1 and 3 in TigA and Hap_1 in Ura3. In case of mitochondrial genes, Hap_2 in C ox2 and Hap_2 and 3 in Nad5.

6.3.3. Phylogenetic analysis

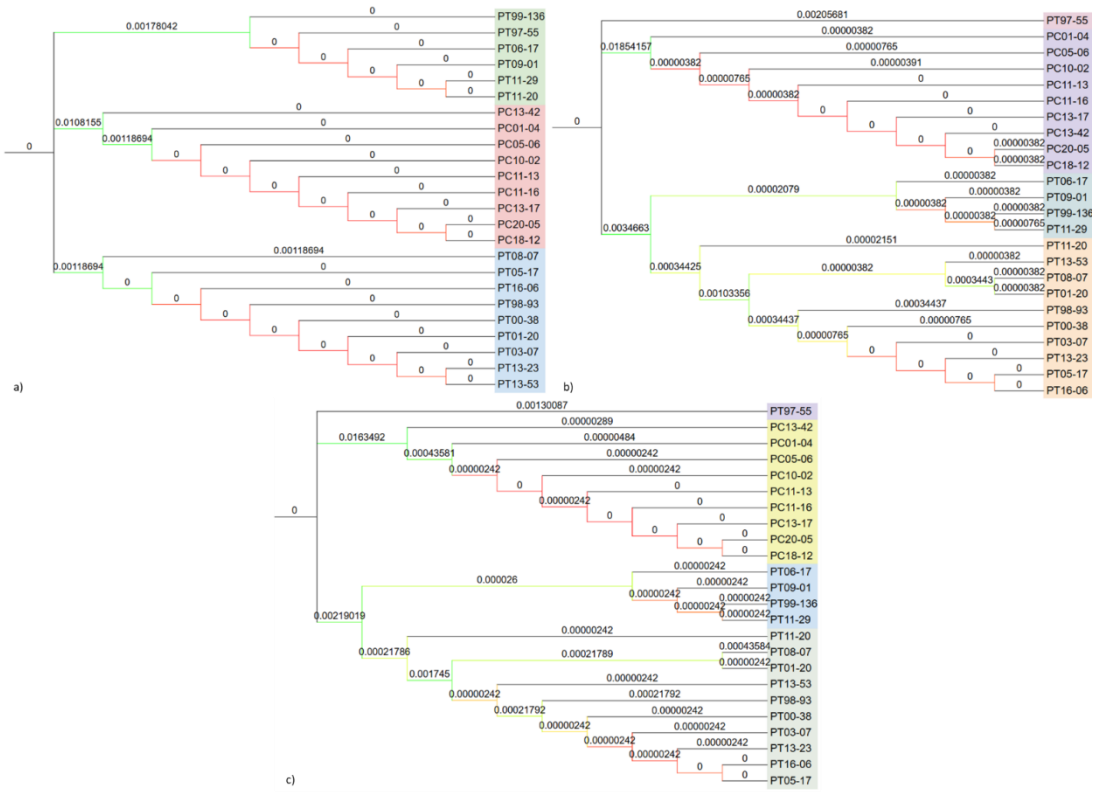
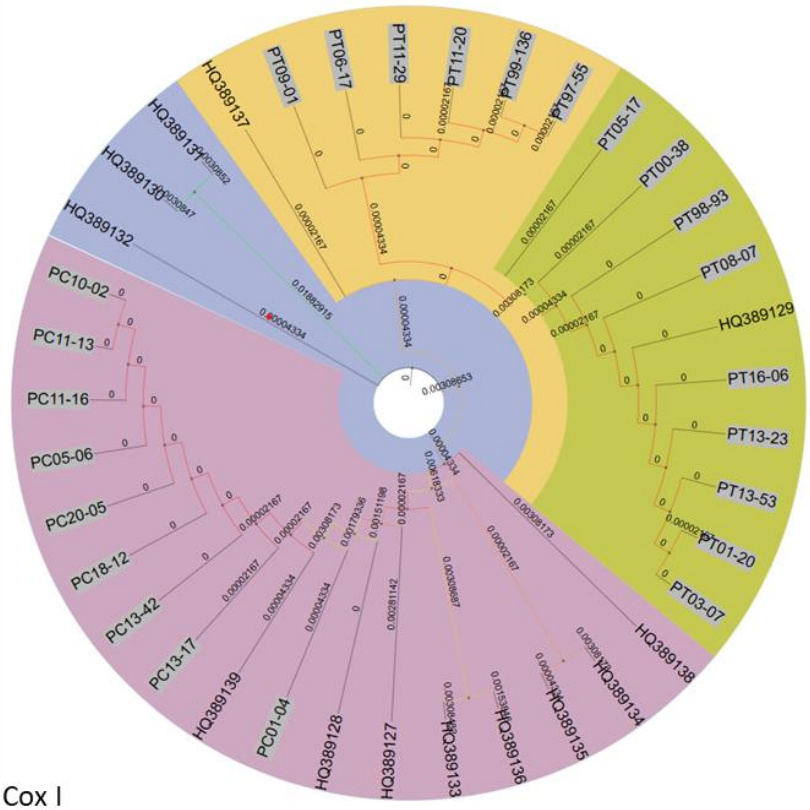
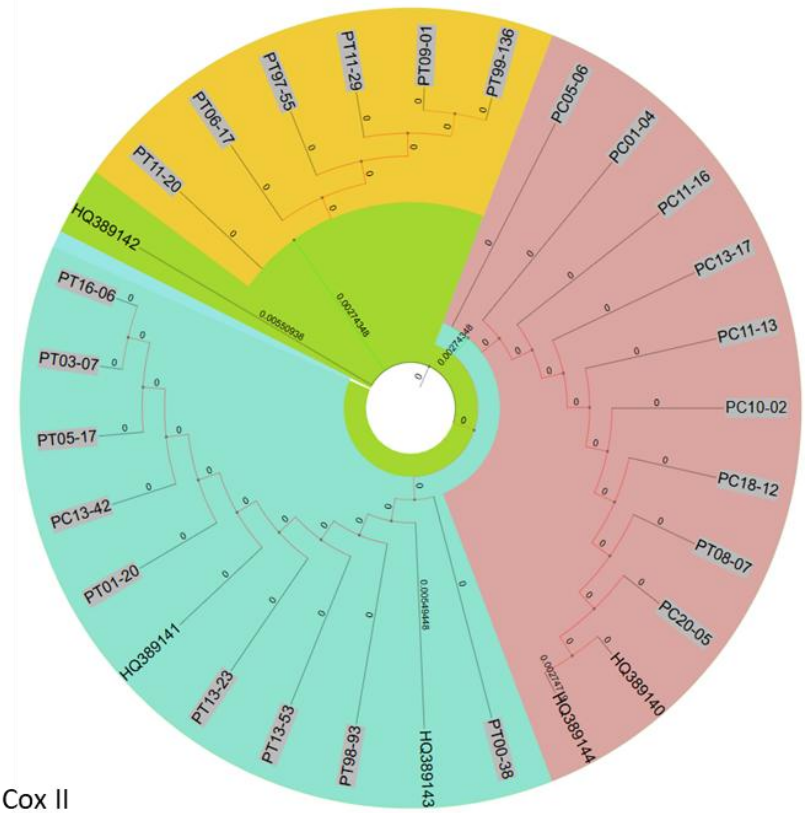


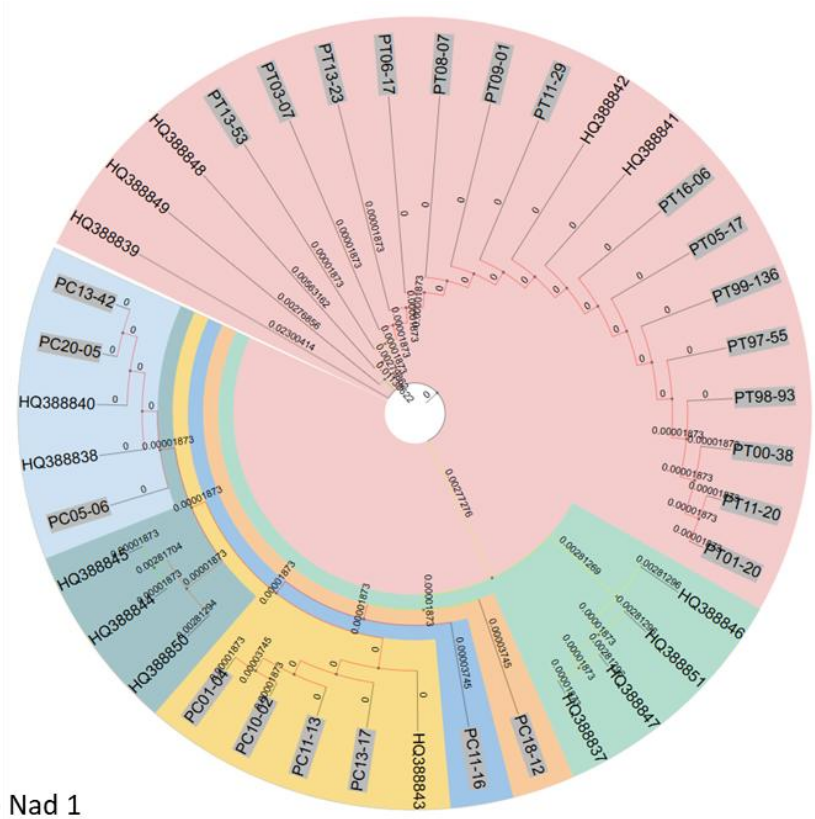
Fig. 6.6. Maximum Likelihood Tree constructed for a) Mitochondrial genes; b) Nuclear genes; c) Combination of a and b. Values on the branch represents the branch length and the clusters were denoted using colours. The colour of the branch corresponds to the bootstrap support with green being the maximum and red denotes minimum support



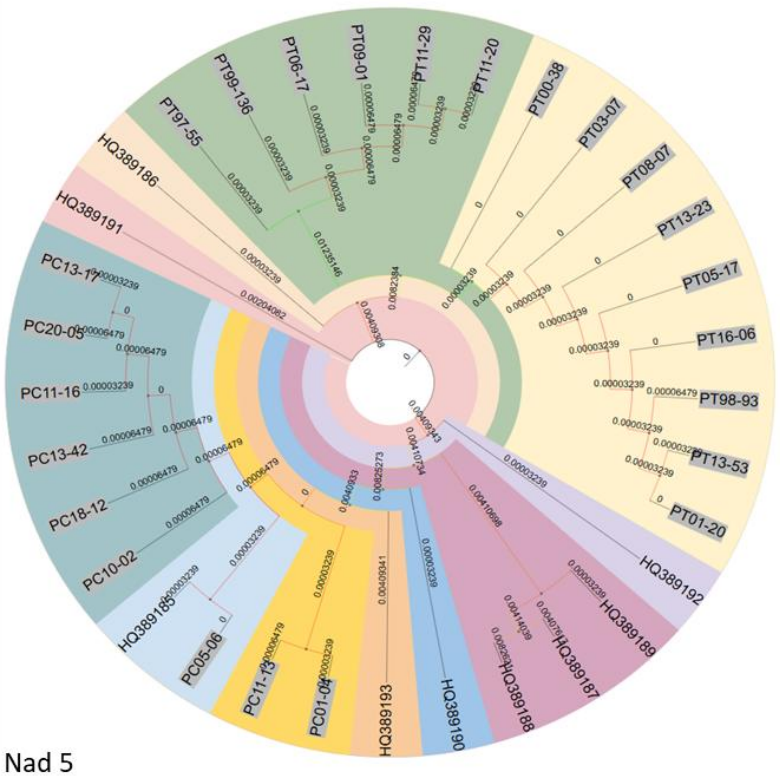
a) Cox I



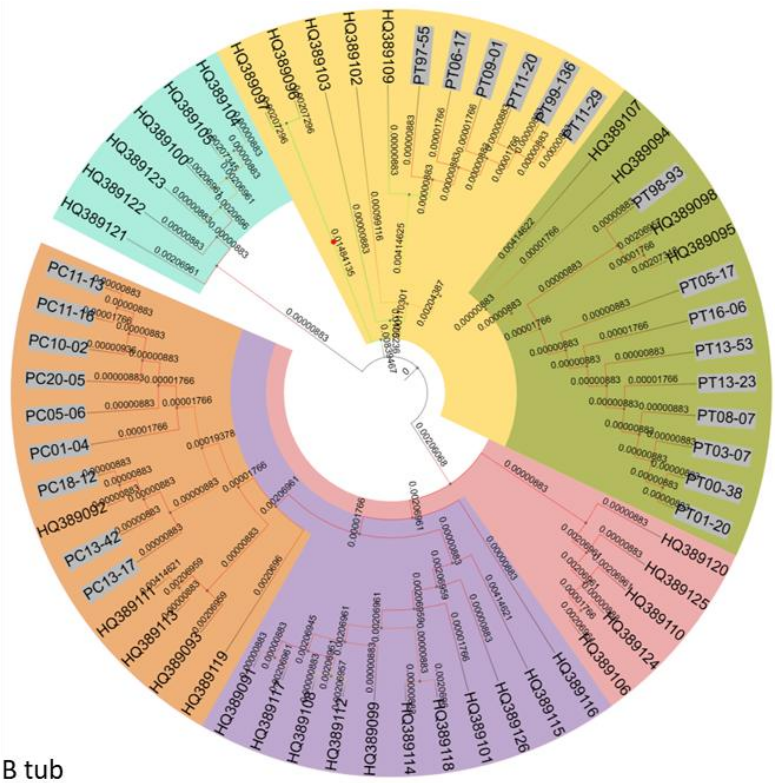
b) Cox II


PT13-53
PT03-07
PT13-23
PT06-17
PT08-07
PT09-01
PT11-29
HQ388842
HQ388841
PT16-06
PT05-17
PT99-136
PT97-55
PT98-93
PT00-38
PT11-20
PT01-20
HQ388846
HQ388851
HQ388847
HQ388837
PC18-12
PC11-16
HQ388843
PC13-17
PC11-13
PC10-02
PC01-04
HQ388850
HQ388844
HQ388845
PC05-06
HQ388838
HQ388840
PC20-05
PC13-42
HQ388839
HQ388849
HQ388848

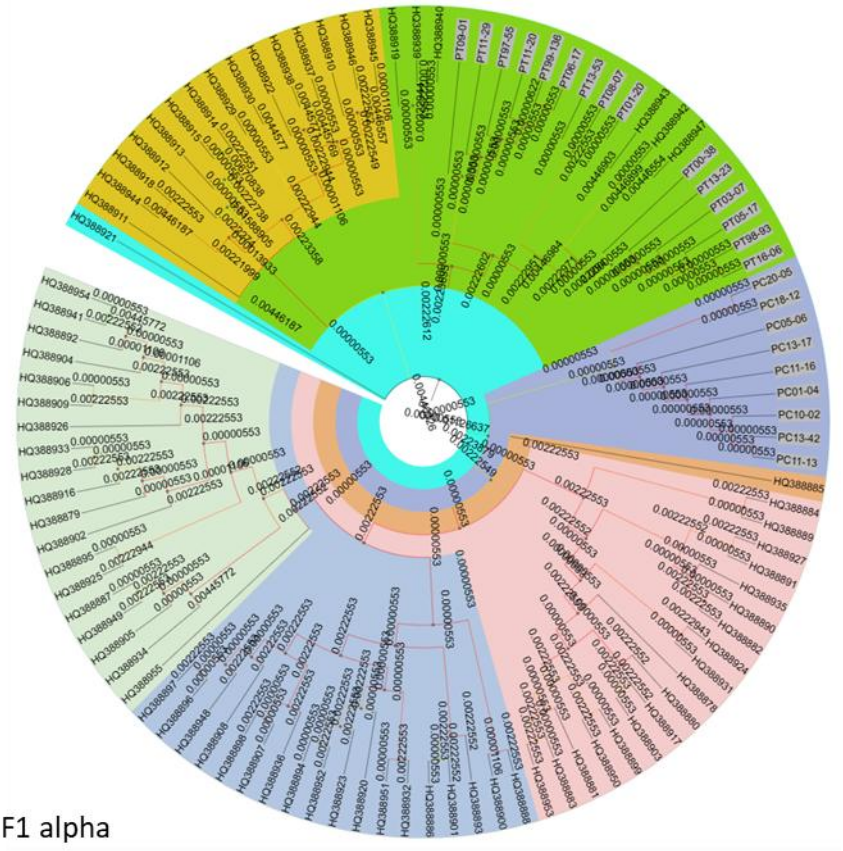
c) Nad 1


PT99-136
PT06-17
PT09-01
PT11-29
PT11-20
PT00-38
PT03-07
PT08-07
PT13-23
PT05-17
PT16-06
PT98-93
PT13-53
PT01-20
HQ389192
HQ389189
HQ389187
HQ389188
HQ389190
HQ389193
PC01-04
PC11-13
PC05-06
HQ389185
PC10-02
PC18-12
PC13-42
PC11-16
PC20-05
PC13-17
HQ389191
HQ389186
PT97-55

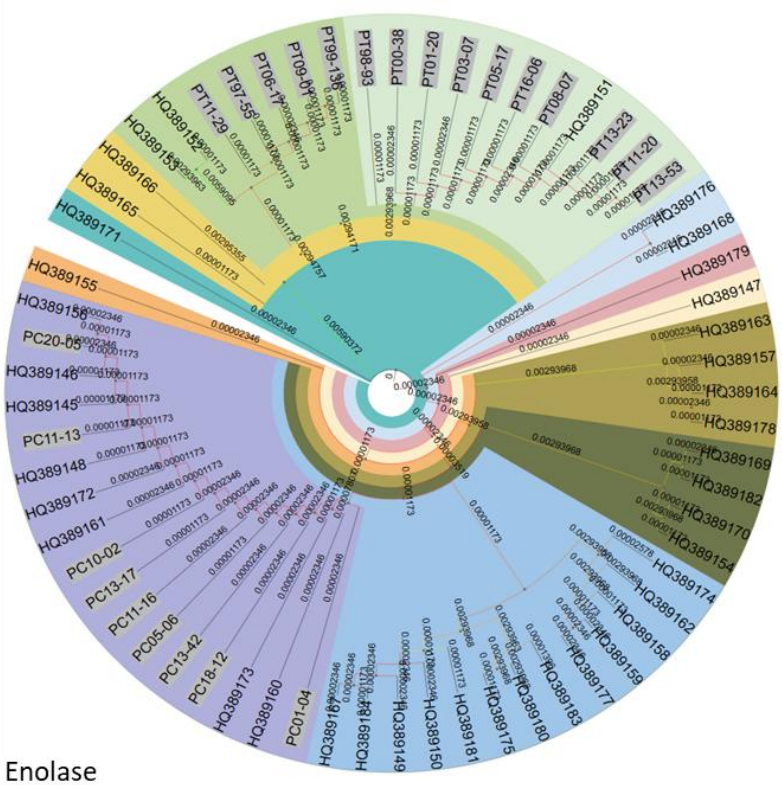
d) Nad 5



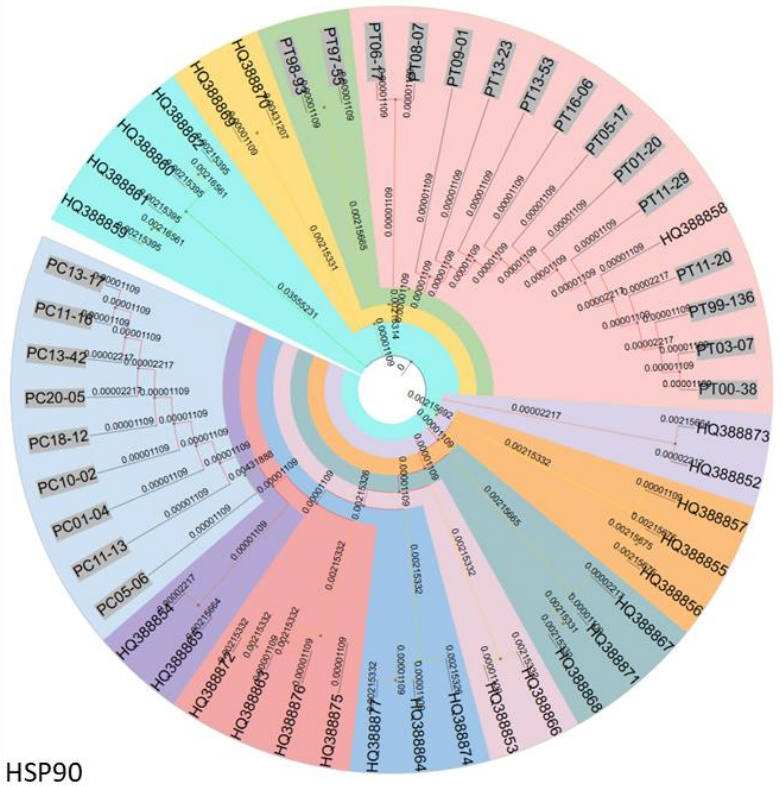
e) B tub



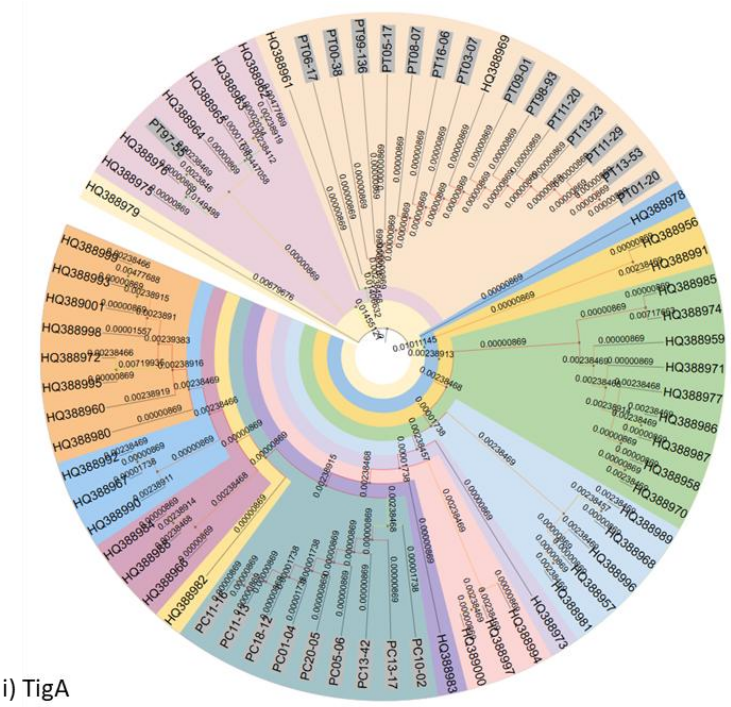
f) EF1 alpha



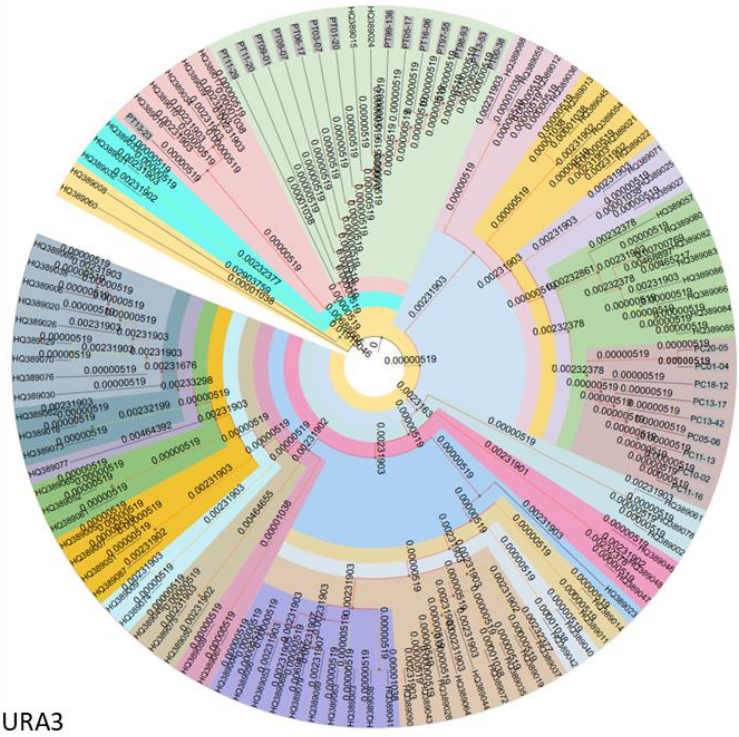
g) Enolase



h) HSP90



i) TigA



j) URA3

Fig. 6.7. Maximum Likelihood Tree with black pepper isolates (highlighted) and isolates from diverse hosts (Quesada-Ocampo *et al.,* 2011b), branch length was displayed and bootstrap support is indicated using red (minimum) and green (maximum) colours a-d) Mitochondrial genes; e-j) Nuclear genes

From the Phylogenetic tree, it was evident that a slight variation was observed among the isolates. *P. capsici* and *P. tropicalis* isolates were clearly differentiated into separate clusters (Fig. 6.6). This observation was similar in the case of concatenated mitochondrial and nuclear genes as well as in the combination of both. Tree constructed with black pepper isolates and isolates from diverse host (Quesada-Ocampo *et al.,* 2011b) (Fig. 6.7), the complete picture of each gene on the whole shows that the diverse host population placed towards the node of the tree and the black pepper isolates have emerged from the above population as these are grouped towards the terminal node of the phylogenetic tree.

6.4. Discussion

The present study broadly targeted the analysis of morphological characters and genetic variation. The morphological characters studied were examined to differentiate the two species, but it was evident from the morphological studies of black pepper isolates that the distinction between the two closely related species used in the study is challenging. The differentiation seemed to be unreliable due to the variation and overlaps of morphological characters, both intra and interspecific in *Phytophthora* spp. (Anandaraj, 2012). A similar report on *Phytophthora* spp. infecting *Hevea brasiliensis* stated that distinguishing the species based on morphology was difficult and possible (Krishnan *et al.,* 2019).

The study also aimed to disentangle the genetic diversity, haplotype, phylogeny and demographic aspects associated with the mitochondrial and nuclear genes of *Phytophthora* species infecting black pepper. Haplotype analysis was performed for the black pepper *Phytophthora* isolates and the isolates from diverse hosts from Hawaii and some of the contiguous United States (Quesada-Ocampo *et al.,* 2011b). A considerable number of variations were identified among the mitochondrial and nuclear genes analyzed in the present study. None of the mutations in the mitochondrial genes were synonymous, whereas only synonymous changes were identified in Beta-tubulin and HSP90. The rest of the changes were non-synonymous, which might have driven evolution by conferring added benefits to the organism with its better adaptability (Andersen *et al.,* 2023; Arenas *et al.,* 2018).

Haplotype diversity was found to be greater for all the genes except for Nad1 and Ur3, where it was moderate. Nucleotide diversity was found to be low for Cox1, Cox2, Nad1, EF1 alpha, enolase and HSP90, whereas it was moderate for Nad5, Beta-tubulin, TigA and Ura3. The mutation rate per site of the population is higher for all the genes, which indicates that the population is undergoing expansion after a severe bottleneck (Garg & Mishra, 2018; Mendez-Harclerode *et al.,* 2007; Song *et al.,* 2014). The rate of recombination that occurs in this population was found to be low to moderate, suggesting that the recombination events were not frequent (Didelot & Maiden, 2010).

Tajima's D, Fu and Li's D and F tests were computed to identify the compliance with the neutral theory of molecular evolution (Tajima, 1989), which states that among the black pepper isolates, the fitness is not affected by the variation at molecular level, but are described by random processes (Duret, 2008). Positive values of Tajima's D were obtained, which points to the fact that low-frequency polymorphisms were less common (Carlson *et al.,* 2005; Eckshtain-Levi *et al.,* 2018) among the Indian population of black pepper isolates. Positive values of Fu and Li's D and F show balancing selection (Tapaopong *et al.,* 2023). Similarly, in the case of *P. infestans* population from Norwegian, the positive values of the Neutrality test indicated the presence of excess of alleles with high frequency (Stefańczyk *et al.,* 2018), and Gómez-Alpizar *et al*. (2007) reported that it is an indication of variation with intermediate frequency. The evolution of RxLR genes of *P. infestans* from various historic herbarium specimens points towards natural selection as indicated by a negative Tajima's D (Coomber *et al.,* 2024).

It was found that haplotypes of all the genes except EF-1α gene of black pepper isolates cluster with the haplotype of the isolates from diverse hosts worldwide. Whereas, for Cox2, Nad5, Enolase, HSP90, TigA and Ura3 unique haplotypes were also identified. In the case of EF-1α, all the haplotypes identified were unique. The isolates belonging to Haplotype 1 of all the genes were the same, except in the case of Cox2 (PT08-07 instead of PC13-42). The haplotypes were distributed randomly, there was no significant correlation between the place of collection of the isolate and

the identified haplotype cluster, which denotes a possibility of gene flow. Whereas in case of *P. infestans* population of China and India, the geographic association with the grouped haplotype was clear suggesting restricted gene flow (Wang *et al.,* 2020).

The phylogenetic and PopART analyses have indicated the presence of the same haplotypes in both Hawaii and some of the contiguous United States and South Indian populations, which typically shows the possibility of gene flow that has occurred between the populations. The phylogenetic analyses of each of the mitochondrial and nuclear genes constructed with the Indian and Hawaii and some of the contiguous United States populations show no clear distinction based on the geographical origin of the isolates in almost all the trees, which points towards the possibility of migration. A similar observation was reported by Mammella *et al*. (2013) where *P. nicotianae* isolates infecting citrus from various geographical locations were grouped in a single clade. However, as pointed out by Quesada-Ocampo *et al*. (2011a), the delineation of the migratory pathway of *P. capsici* calls for an investigation into its phylogeny and evolution. Nevertheless, the prevalence of *P. infestans* in India was explored extensively and traced four migratory events (Dey *et al.,* 2018; Patarroyo *et al.,* 2024). On contrary, a report from China which analyzed the gene flow in *P. infestans* population between India and China based on eEF-1α gene haplotypes found restricted gene flow (Wang *et al.,* 2020). Furthermore, the migration in *P. colocasiae* populations among different fields within India was reported by Nath *et al*. (2013). The migration of necrotrophic or hemibiotrophic plant pathogens is often restricted on its own and is attributed to trade or human migration (Miedaner & Garbelotto, 2024).

6.5. Conclusion

The area of tracing the evolutionary history of *P. capsici* and *P. tropicalis* in India was poorly explored. The current study provided insight into the evolutionary background of the population of *Phytophthora* spp. infecting black pepper in India. Demographic analysis revealed post-bottleneck expansion and balancing selection, which explains that when the genetic diversity is reduced during a bottleneck, balancing selection facilitated the restoration of genetic variation. Thereby

improving the adaptability and resilience of the population by maintaining the advantageous alleles through balancing selection. It was evident from the phylogenetic study that the black pepper isolates from India traced back to Hawaii and some of the contiguous United States population.

CHAPTER 7

ANALYSIS OF RXLR EFFECTORS IN *PHYTOPHTHORA CAPSICI* AND *P. TROPICALIS* INFECTING BLACK PEPPER

Abstract

Phytophthora has a vast array of effector molecules that help attack its hosts. These effector molecules ultimately act by suppressing various immune responses exhibited by the host. The present study aimed to analyze the expression of the selected RxLR effectors of *P. capsici* and *P. tropicalis,* i.e., RxLR11, RxLR15, RxLR22, RxLR29, RxLR48, RxLR103, RxLR113, RxLR132, RxLR207 and RxLR305 during *Phytophthora* infection in a susceptible variety of black pepper, Sreekara. All the RxLR effectors, except RxLR11 showed upregulation during pathogen infection. Especially, RxLR29 and RxLR132 were highly expressed at an early stage of infection. Hence, these two RxLR effector genes from *P. capsici* and *P. tropicalis* isolates were cloned, sequenced, and the three-dimensional structures of these two effectors were predicted using homology modelling. It was found that the simulated structures were within the accepted range. Further, based on the sequence similarity with the earlier reported RxLR effectors, protein-protein interaction of RxLR29 and RxLR132 with DRB4 and CMPG1, respectively was carried out to find out their possible mode of action. Docking studies showed the presence of biologically significant interactions between RxLR29 and DRB4 and RxLR132 and CMPG1.

7.1. Introduction

Black pepper (*Piper nigrum*) is an indigenous crop of Kerala also known as 'Black gold' is a crop with major historic significance due to its high trade value and also has a prime role in the global market, hence any kind of stress (either biotic or abiotic) in its cultivation is a serious cause of concern. One of the most serious biotic stresses is the one that is imparted by the plant pathogens. This chapter focuses on one of the aspects of infection caused by the oomycete *P. capsici* and *P. tropicalis* on black pepper with an emphasis on the study of RxLR effector molecules.

Unlike the animal immune system, plants mainly depend on the innate immune system to defend the pathogens, which are mainly of two types: the first one is MAMP (microbe-associated molecular pattern) and PAMP (pathogen-associated molecular pattern) triggered immunity (PTI); the second one is effector-triggered immunity (ETI). The plant uses Pattern Recognition Receptors (PRRs) on the cell surface for perceiving the damage-associated molecular patterns (DAMPs) and MAMPs (Han, 2019). Another type of molecule is R proteins, which identify the effectors encoded by the avr genes of the pathogen (gene-for-gene hypothesis) (van Wersch *et al.,* 2019).

As far as an oomycete pathogen namely *Phytophthora* is concerned, being a hemi biotrophic pathogen, it has a biotrophic phase where the effectors were released via haustoria to suppress the plant immune response and a necrotrophic phase, where proteins and toxins which aids in cell death were synthesized (Wang *et al.,* 2023). The two major types of effectors include apoplastic effectors, which act in the extracellular space and cytoplasmic effectors, which are targeted into the plant cells (Lovelace *et al.,* 2023). The present study deals with the cytoplasmic effectors, which have a conserved region comprised of an N-terminal followed by amino acid arginine, any amino acid, leucine and arginine (RxLR) motif, which targets specific proteins destined for a specific function (Li *et al.,* 2022). *Phytophthora* adopts several mechanisms to overcome the resistance conferred by the host R genes like a mutation in one or two amino acids of the RxLR gene which makes it

unrecognizable by the host R protein, frameshift mutations, which produces the truncated protein which is again unrecognizable by the host (Du *et al.,* 2018).

Here we have studied the expression levels of ten different RxLRs following the infection of *Phytophthora* in black pepper, and two of the highly expressed RxLRs, i.e., RxLR29 and RxLR132 were further focused. Based on the earlier reports, the former has maximum similarity with PSR2 (suppressors of RNAi called *Phytophthora* Suppressor of RNA Silencing 2) of *P. sojae*, which associates with double-stranded RNA binding protein 4 (DRB4), which in turn binds with Dicer-like 4(DCL4) and digests the dsRNA. Hence, bypassing the siRNA-mediated defence response of the host (Hou *et al.,* 2019). Whereas, the latter which is a homologue of AVR3a of *P. infestans* gets involved in the stabilization of U-box E3 ligase of CMPG1, which naturally has a role in substrate and self-degradation by 26S proteasome. Therefore, during the biotrophic phase, the AVR3a is up-regulated and gets down-regulated toward the necrotrophic phase, thereby controlling the cell death to benefit the infection process (Gilroy *et al.,* 2011).

7.2. Methodology

7.2.1. Source of cultures and its maintenance

The source of isolates and their maintenance procedures were given in Chapter 6 (Section 2.1). The isolates used for the study are listed in Table 7.1. The identity of the isolates was confirmed using the Ypt gene (Jeevalatha *et al.,* 2021) and ITS-based primers (Bhai *et al.,* 2022).

Table 7.1. List of selected *P. capsici* and *P. tropicalis* isolates

Sl. No.	Species	Name of isolate	Place of collection
1	*P. capsici*	01-04	Adivaram, Kozhikode
2	*P. capsici*	05-06	Peruvannamuzhi, Kozhikode
3	*P. capsici*	18-02	Plot 1, Kozhikode
4	*P. capsici*	18-12	Idukki
5	*P. capsici*	20-05	Panniyur, Kannur
6	*P. capsici*	21-02	Panniyur, Kannur
7	*P. capsici*	22-03	Maharashtra
8	*P. capsici*	23-01	Peruvannamuzhi, Kozhikode
9	*P. tropicalis*	97-55	Valparai, Coimbatore
10	*P. tropicalis*	98-93	Adyanadka, Dakshin Kannada
11	*P. tropicalis*	03-07	Adivaram, Kozhikode
12	*P. tropicalis*	06-17	Rasi Estate, Yercaud, Salem
13	*P. tropicalis*	09-01	Peruvannamuzhi, Kozhikode
14	*P. tropicalis*	11-29	Wayanad
15	*P. tropicalis*	13-23	Nedumkandam, Idukki
16	*P. tropicalis*	13-53	Goa

7.2.2. In vitro inoculation and RT-PCR

Three-day-old culture discs of PC05-06 were kept for sporulation in sterile distilled water at 25±1°C under continuous illumination for 48h. The discs were examined for the development of sporangia using Leica DM 5000 B microscope (Leica Mikrosystems Vertrich GmbH, Germany) and the sporulated discs were induced to release the zoospores by applying a cold shock, i.e., the sporulated discs were incubated at 4°C for 30 minutes followed by a 30 minutes incubation at room temperature. After confirming the presence of zoospores using the microscope, about 200µL of the suspension was inoculated by spraying onto the detached leaves of a susceptible variety of black pepper Sreekara and were incubated in a humid chamber at 25±1°C. Those leaves sprayed with sterile distilled water served as a control, and the assay was performed in triplicate. The leaves were collected at an interval of 0, 2, 4, 6, 8, 16, 24 and 36 h after inoculation. The total RNA was isolated from the above sample using a NucleoSpin RNA Plant and Fungi kit (Macherey-Nagel Inc, Allentown, USA) by following the manufacturer's instructions. The RNA samples were quantified using DS-11+ Spectrophotometer (DeNovix®) and were used to synthesize cDNA using RevertAid First Strand cDNA Synthesis Kit

(Thermo Scientific). The reaction mixture of 20µL composed of QuantiFast SYBR green PCR Master mix 10µL, 1µL each of forward and reverse primers (Table 7.2) and 0.5µL of 1µg/µL template. Template samples with no reverse transcriptase (NRT) and a non-template control were also included. The actin gene of *P. capsici* was used as a reference gene. qPCR was performed in Rotor-Gene 2000 Real Time Cycler (Qiagen Germany) by following a temperature profile of 95°C for 5 minutes and 40 cycles at 95°C for 10s and 60°C for 30s. Melt curve and relative expression of all the RxLR were analyzed

Table 7.2. List of PCR and qPCR primers used in the study

Sl. No.	Primer	Sequence
1	Pc-LR29-FP	5'-ATGCGTCTTCATTGTATCGTTGTG-3'
	Pc-LR29-RP	5'-CTACCAAACACATCGTCCGCC-3'
2	RxLR132-FP	5'-ATGCGCCTGTCCTTCCTGCTG-3'
	RxLR132-RP	5'-TTACACATAATCCCTATAGGTCAT-3'
3	PcActin-F	5'-CCCATCGAGGGTTACGC-3'
	PcActin-R	5'-CCGTGGTGGTGAACGAGTAA-3'
4	LR11-QFP	5'-CATTGTTGCCCTGACCGTTG-3'
	LR11-QRP	5'-CCCTCTCTTCGTCCTCCGTA-3'
5	LR15-QFP	5'-GCAGCTAGCGAAGCGAATAC-3'
	LR15-QRP	5'-TCCATCAGCTTCATCCTTGA-3'
6	LR22-QFP	5'-TACGCAATCTCGTGTTCCTG-3'
	LR22-QRP	5'-ACGATCTTGTCCAGCAGTCC-3'
7	LR29-QFP	5'-AAGACGTATTCGGACGAGGC-3'
	LR29-QRP	5'-GGTCCTTCAGCCAAAGACGA-3'
8	LR48-QFP	5'-GAGGAGAGGGGATGGTTCTC-3'
	LR48-QRP	5'-TGATCTGTGCTGGAGTGAGG-3'
9	LR103-QFP	5'-TTCGCTTTCCTTCTGTTCGT-3'
	LR103-QRP	5'-TGTTCGTCTGGTGGTGAGAG-3'
10	LR113-QFP	5'-CGTCGTCGCTCTTCTCTTCA-3'
	LR113-QRP	5'-CCGCTTCACCTCGAAGAACT-3'
11	LR132-QFP	5'-GAAGAAGCCGACAGAGAGGA-3'
	LR132-QRP	5'-TAATTGCCTTGGCCATTTTC-3'
12	LR207-QFP	5'-GCACATATTGGCTTCGCGTT-3'
	LR207-QRP	5'-AATACCTCAGTTGCCAGGGC-3'
13	LR305-QFP	5'-CTTCGAGAACCAGCACCAGT-3'
	LR305-QRP	5'-GCATGCGGAACTTAGTAGGC-3'

7.2.3. RxLR gene amplification and elution of the amplicons

The DNA extraction protocol was described in Chapter 6 (section 2.3). PCR amplification of the selected isolates (Table 7.1) was carried out in a 20 µL reaction volume containing 10 µL of Emerald Amp GT PCR Master Mix (2X Premix), 0.5 µL each of forward and reverse primers (Table 7.2) and 100ng/µL of DNA. Thermal cycling was performed in Gradient Thermal Cycler IG-96GEP by following the thermal cycling of an initial denaturation of 10 min at 95^{o}C, followed by 35 cycles of denaturation at 95^{o}C for 1 min, annealing at 60^{o}C for 1 min, extension at 72^{o}C for 1 min, and final extension of 72^{o}C for 10 min. The amplicons were run in 1% agarose gel in TAE buffer at 100 V for 2 hours and visualized under UV transilluminator. The amplicons with 840bp were cut out from the gel and eluted using NucleoSpin® Gel and PCR Clean-up (Macherey-Nagel, Germany) by following the manufacturer's instructions.

7.2.4. Cloning, transformation and selection of transformants

The amplicons were cloned using Thermo Scientific CloneJET PCR Cloning Kit, by following the manufacturer's instructions. Transformation was performed using the TransformAid Bacterial Transformation Kit (Thermoscientific) by following the manufacturer's instructions. The cells were then plated onto an LB agar plate supplemented with Ampicillin, X-Gal and IPTG and incubated overnight at 37^{o}C. The white colonies were screened for the insert by colony PCR. The colonies with insert were inoculated in 5 mL of LB broth supplemented with Ampicillin and incubated overnight at 37^{o}C. Plasmid isolation was performed using the NucleoSpin Plasmid Mini kit for plasmid DNA (Macherey-Nagel, Germany) by following the manufacturer's instructions. The presence of the insert was further confirmed by performing PCR by following the conditions mentioned above. The plasmid was sent for sequencing at Eurofins Genomics Pvt. Ltd, Bangalore.

7.2.5. Sequence analysis

Sequences were edited manually, and multiple sequence alignment was performed using ClustalX2 (Larkin *et al.,* 2007). The nucleotide sequences were converted to

amino acid sequences using the Expasy-translate tool. The amino acid sequences were further aligned using ClustalX2. Signal peptide prediction was performed using SignalP-6.0. Motif in the aligned amino acid sequence was analyzed using MEME Suite 5.5.7 (Multiple Em for Motif Elicitation) (Bailey & Elkan, 1994) with classic discovery mode.

7.2.6. Structure and protein-protein interaction studies

The three-dimensional structure of the amino acid sequences was constructed using an online platform SWISS-MODEL (Waterhouse *et al.,* 2024), where the structure was constructed based on a template with significant similarity from PDB. MolProbity assessment was executed to validate the structural integrity and stereochemistry of the predicted structures. Protein-protein docking studies were run using a software platform HADDOCK 2.4 (High Ambiguity Driven protein-protein DOCKing) (Honorato *et al.,* 2024). The active residues were defined using CASTp (Computed Atlas of Surface Topography of proteins) (Tian *et al.,* 2018) and P2Rank (Jakubec *et al.,* 2022; Jendele *et al.,* 2019; Krivák & Hoksza, 2018). The output files of the best-ranked model were visualized using PyMOL, Version 3.0 (Schrödinger & DeLano, 2020).

7.3. Results

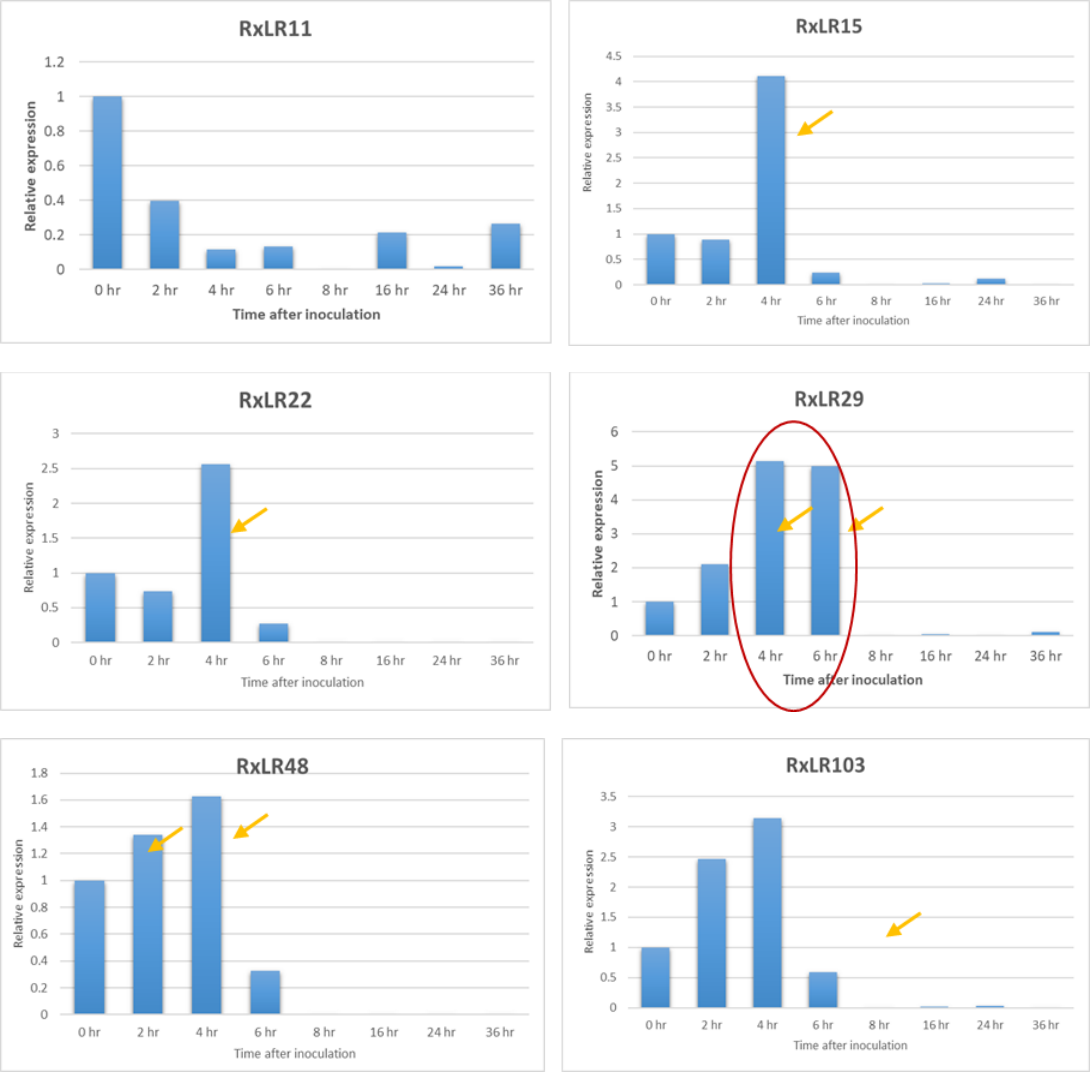
7.3.1. Isolate selection and species identification

A total of sixteen isolates, eight each of *P. capsici* and *P. tropicalis,* collected during the period of 1997 to 2023 were selected after confirming the species identity (Table 7.1).

7.3.2. Expression analysis

A Plethora of effectors was predicted from PC05-06 (*P. capsici*) using an offline tool Effector P 3.0 beta, which includes about 460 cytoplasmic effectors (RxLR and CRN) and 14 apoplastic effectors (NLPs). The relative expression of RxLR genes (RxLR11, RxLR15, RxLR22, RxLR29, RxLR48, RxLR103, RxLR113, RxLR132, RxLR207 and RxLR305) were analyzed analysed at 0, 2, 4, 6, 8, 16, 24 and 48h

after inoculation. It was observed that all the RxLRs except RxLR11 were upregulated during different time intervals post-inoculation. A 2.5 to 4-fold increase in the expression at 4h post-inoculation was observed for RxLR15, RxLR22, RxLR207 and RxLR305. The upregulation persisted from 2 to 4h after inoculation and later decreased in genes such as RxLR48, RxLR103, RxLR113 and RxLR132. More than 5-fold increased expression was observed in RxLR29 after 4 to 6h after inoculation, and exceptionally greater expression among the ten genes was observed for RxLR132, that is a 65-fold increase after 2h and further increased to 125-fold after the 6h of inoculation (Fig. 7.1).



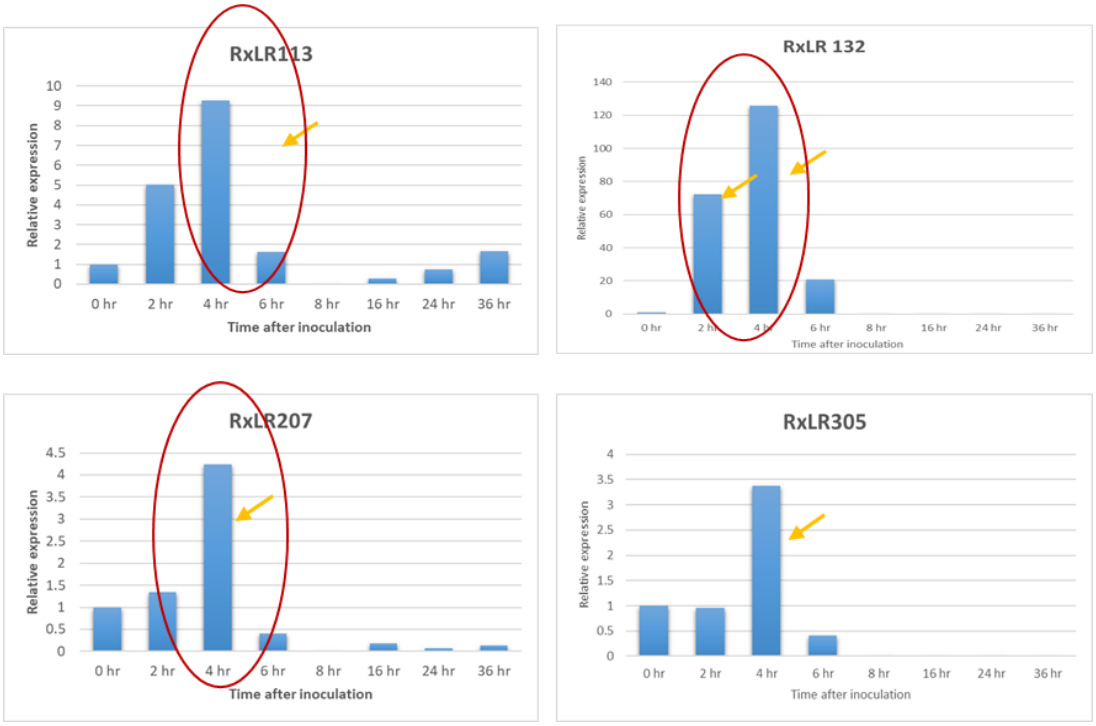


Fig. 7.1. Graphs showing the level of expression of RxLR29 and RxLR132 after challenging with *P. capsici* in vitro

7.3.2. Sequence analysis

Multiple sequence alignment of the nucleotide and amino acid sequences showed that the RxLR29 gene region is highly variable, and variability was also observed in the case of RxLR132, and the mutations observed were mainly non-synonymous, insertions and deletions and these variations were not species-specific. The aligned amino acid sequence showed a signal peptide motif of variable length and RxLR motif, which was followed by a DEER motif in RxLR29 (Figs. 7.2 & 7.3). Unlike RxLR29, only 6 of the samples were amplified in RxLR132. The number of nonsynonymous mutations, insertions and deletions was less and the DEER motif was absent here (Fig. 7.3). The signal peptide region in the amino acid sequence of each isolate, the cleavage site and the mature protein region were clearly depicted in Figures 7.4, 7.5 & 7.6. Conserved motifs were described using the MEME suite (5.5.7) and were represented in different colours, each representing the conserved amino acid sequence (Fig. 7.7) present along the length of the sequence. The Sequence logo of the 3 motifs is given in Figure 7.8 for greater clarity.

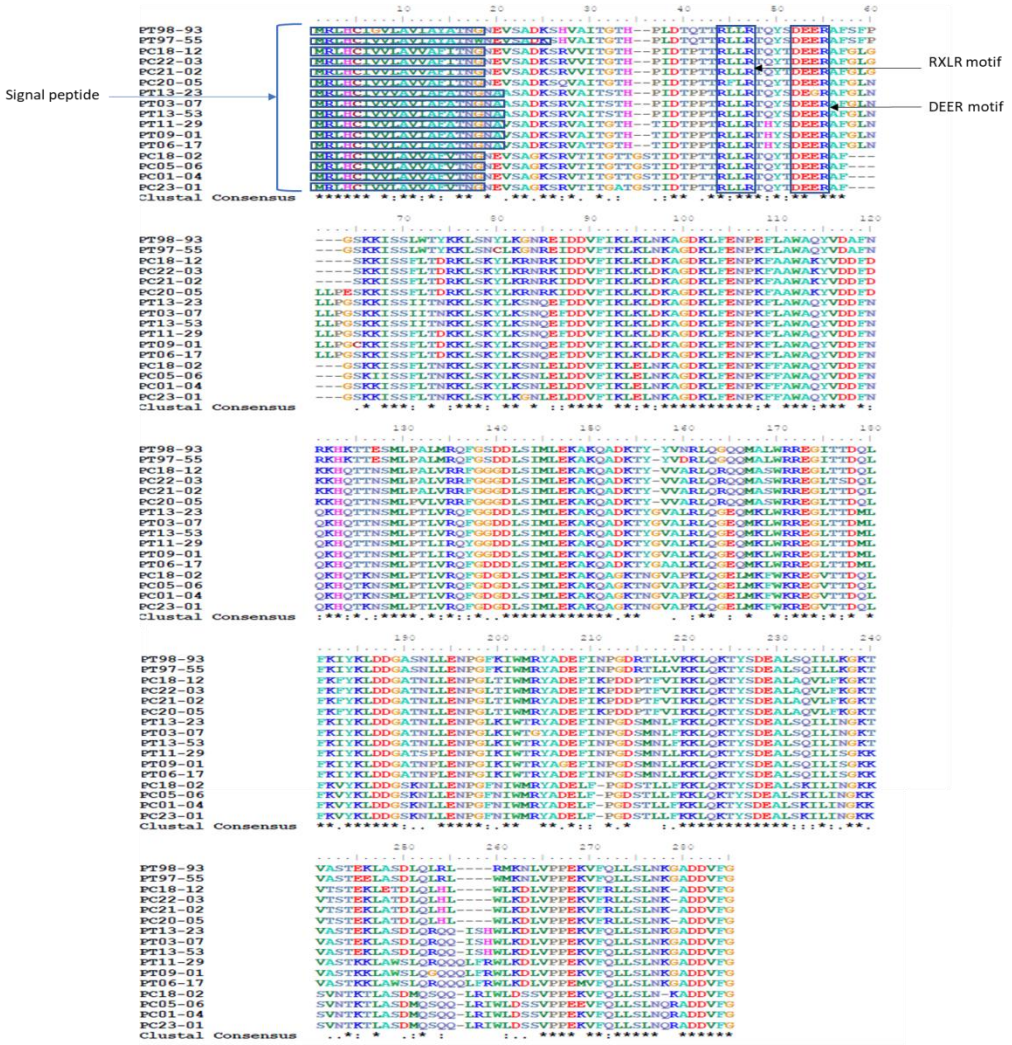


Fig. 7.2. Multiple sequence alignment of RxLR29 gene of *P. capsici* and *P. tropicalis* infecting black pepper

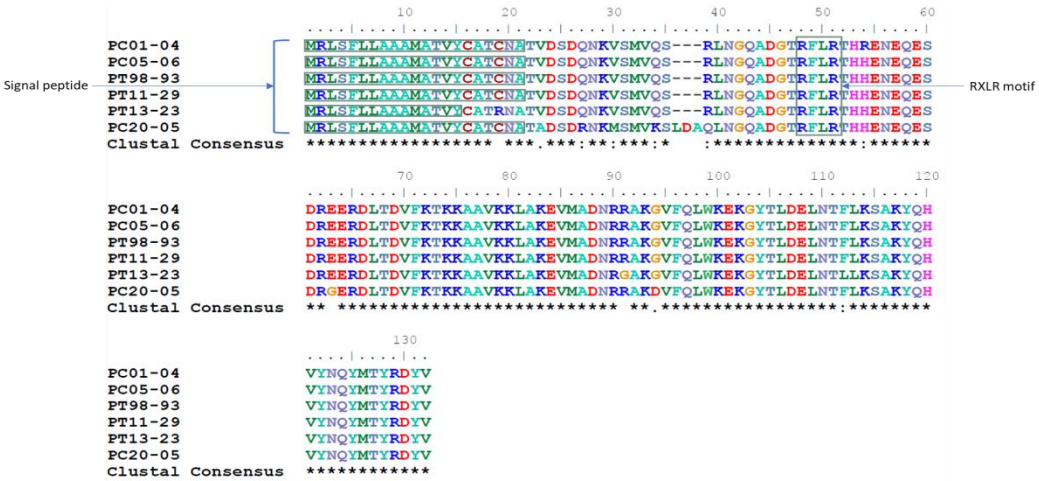


Fig. 7.3. Multiple sequence alignment of RxLR132 gene of *P. capsici* and *P. tropicalis* infecting black pepper

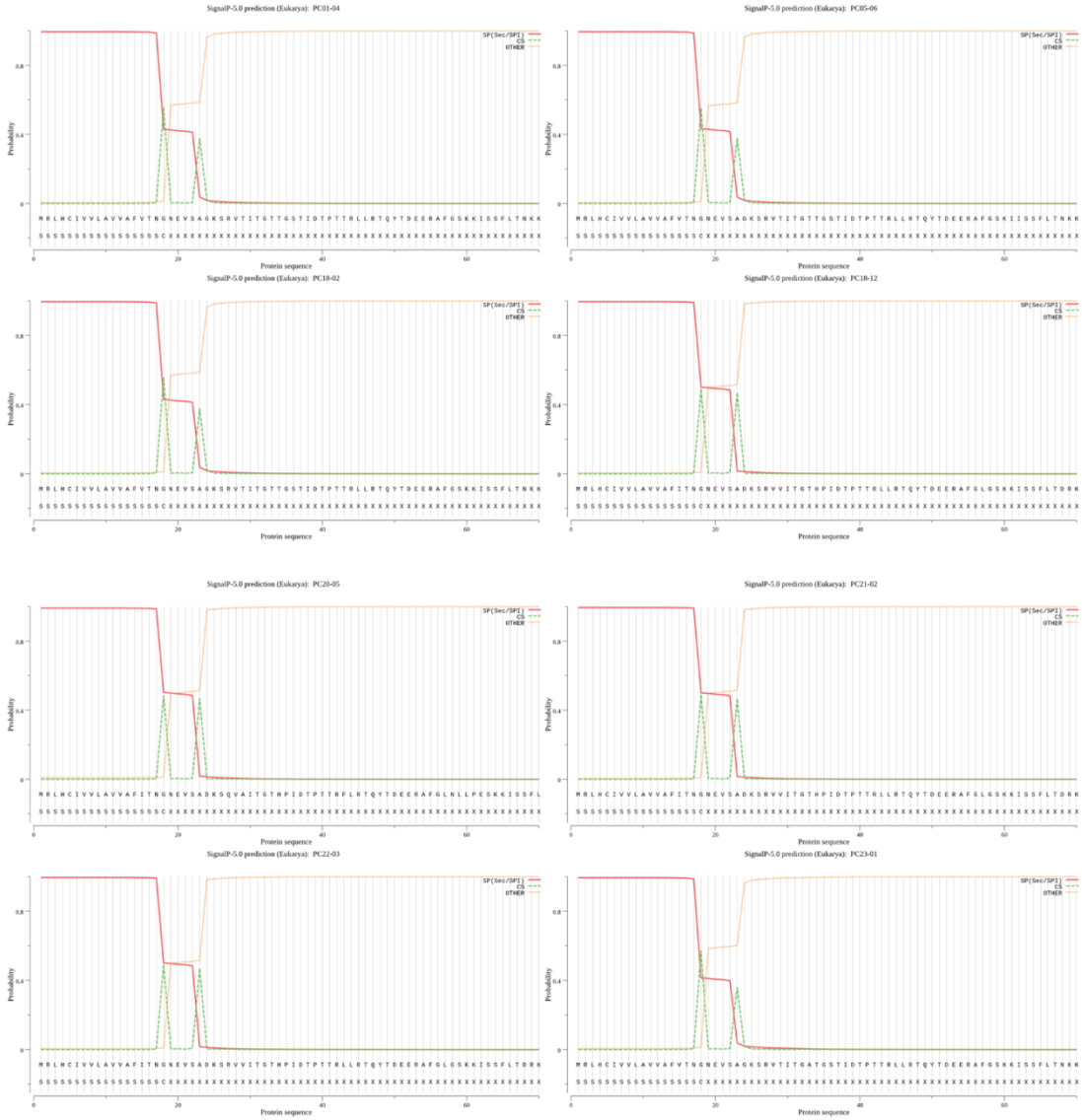


Fig. 7.4. Graph showing the signal peptide region, cleavage site and mature protein in RxLR29 amino acid sequence of *P. capsici* isolates

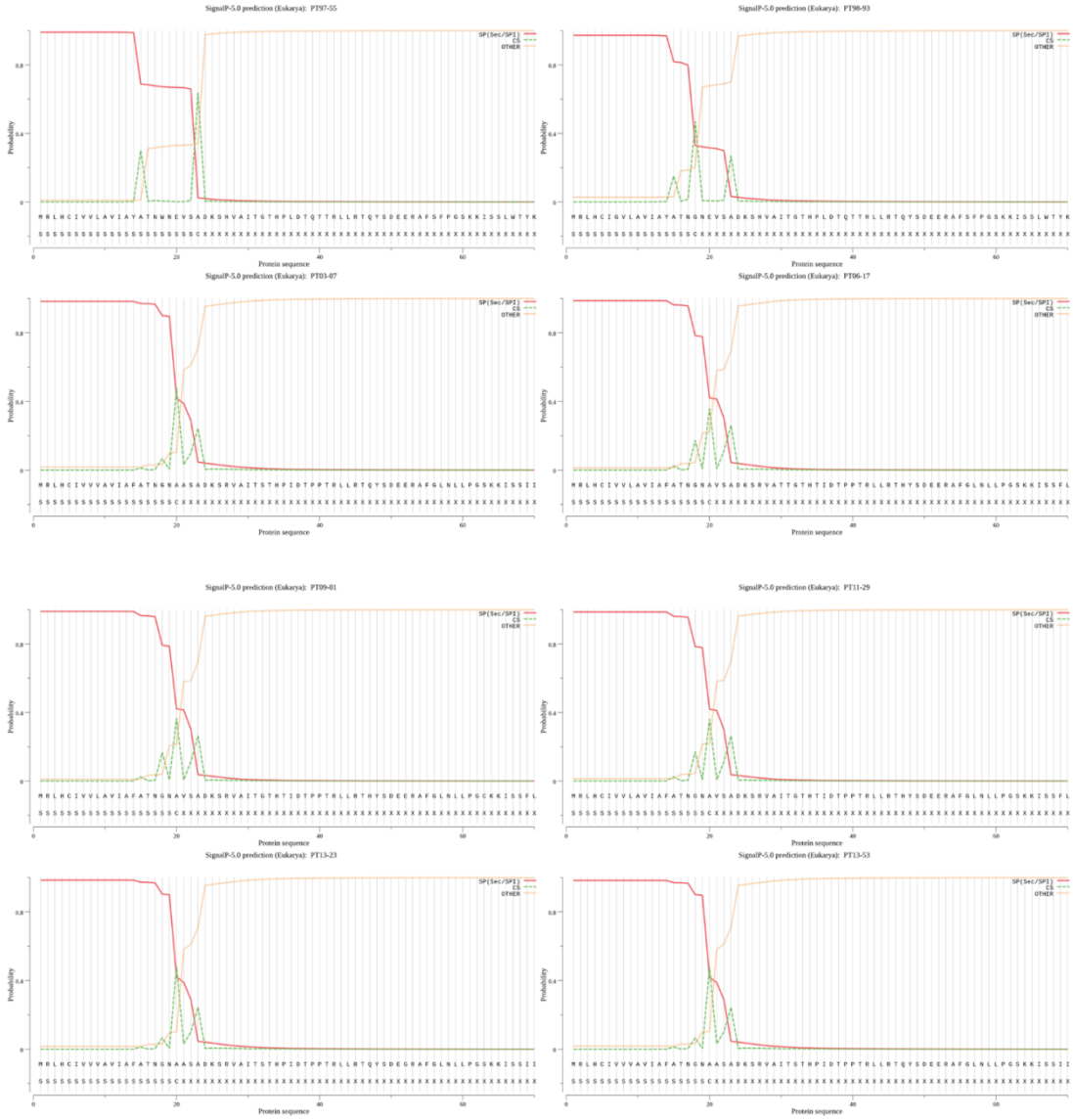


Fig. 7.5. Graphical representation of signal peptide region, cleavage site and mature protein in RxLR29 amino acid sequence of *P. tropicalis* isolates

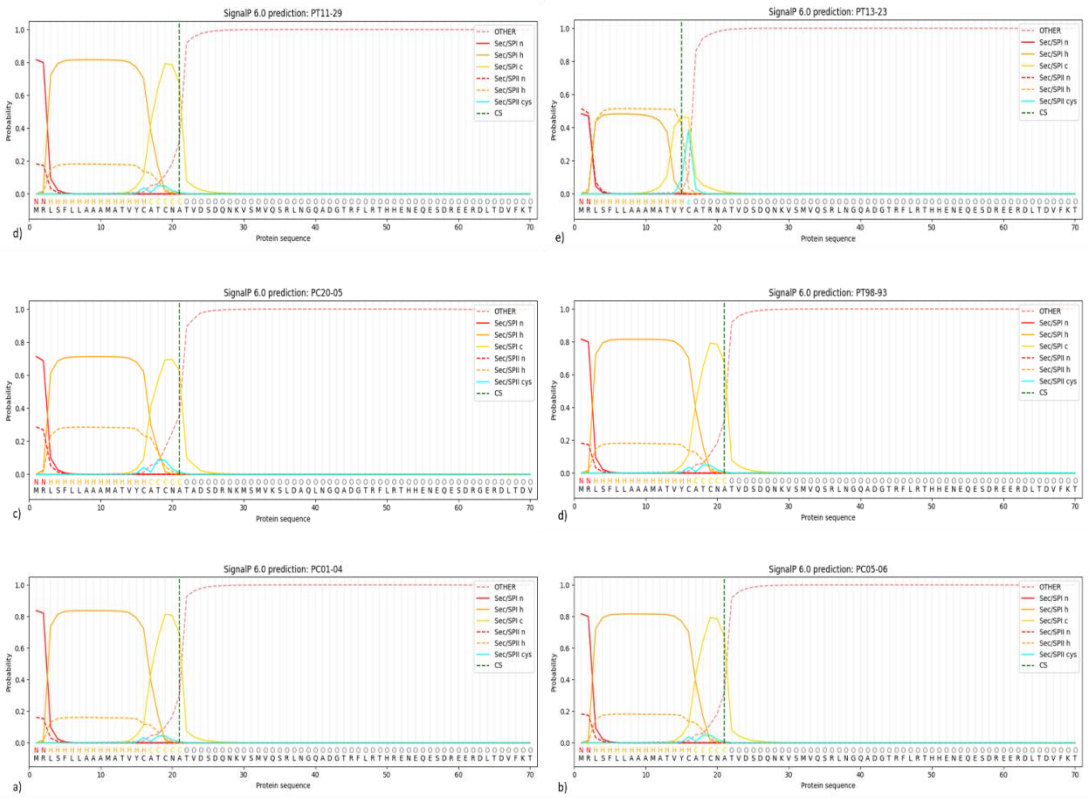


Fig. 7.6. Graphical representation of signal peptide region, cleavage site and mature protein in RxLR132 amino acid sequence of *P. capsici* and *P. tropicalis* isolates

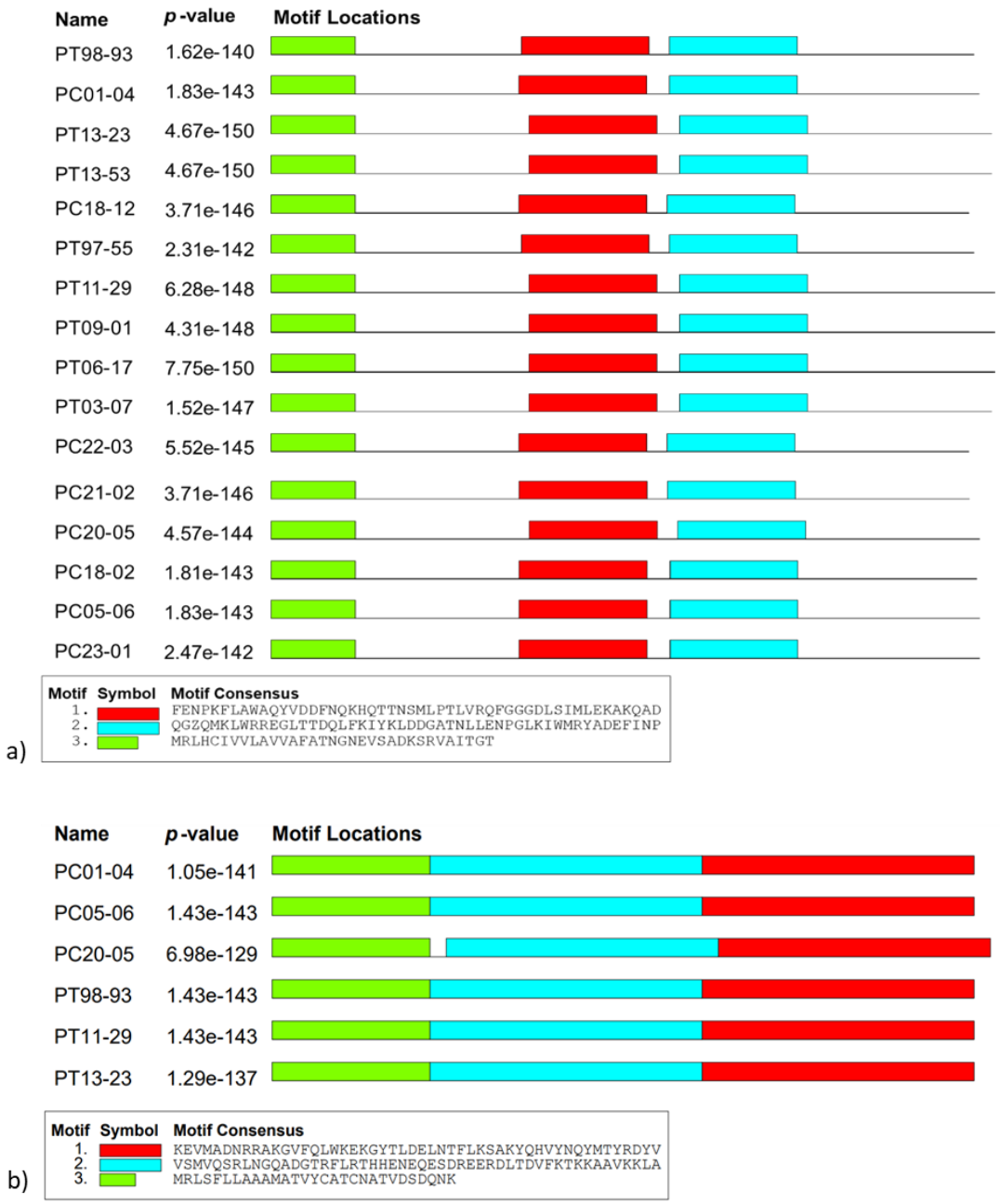


Fig. 7.7. Visual representation of the conserved motifs with each sequence represented with different hues. a) RxLR29; b) RxLR132

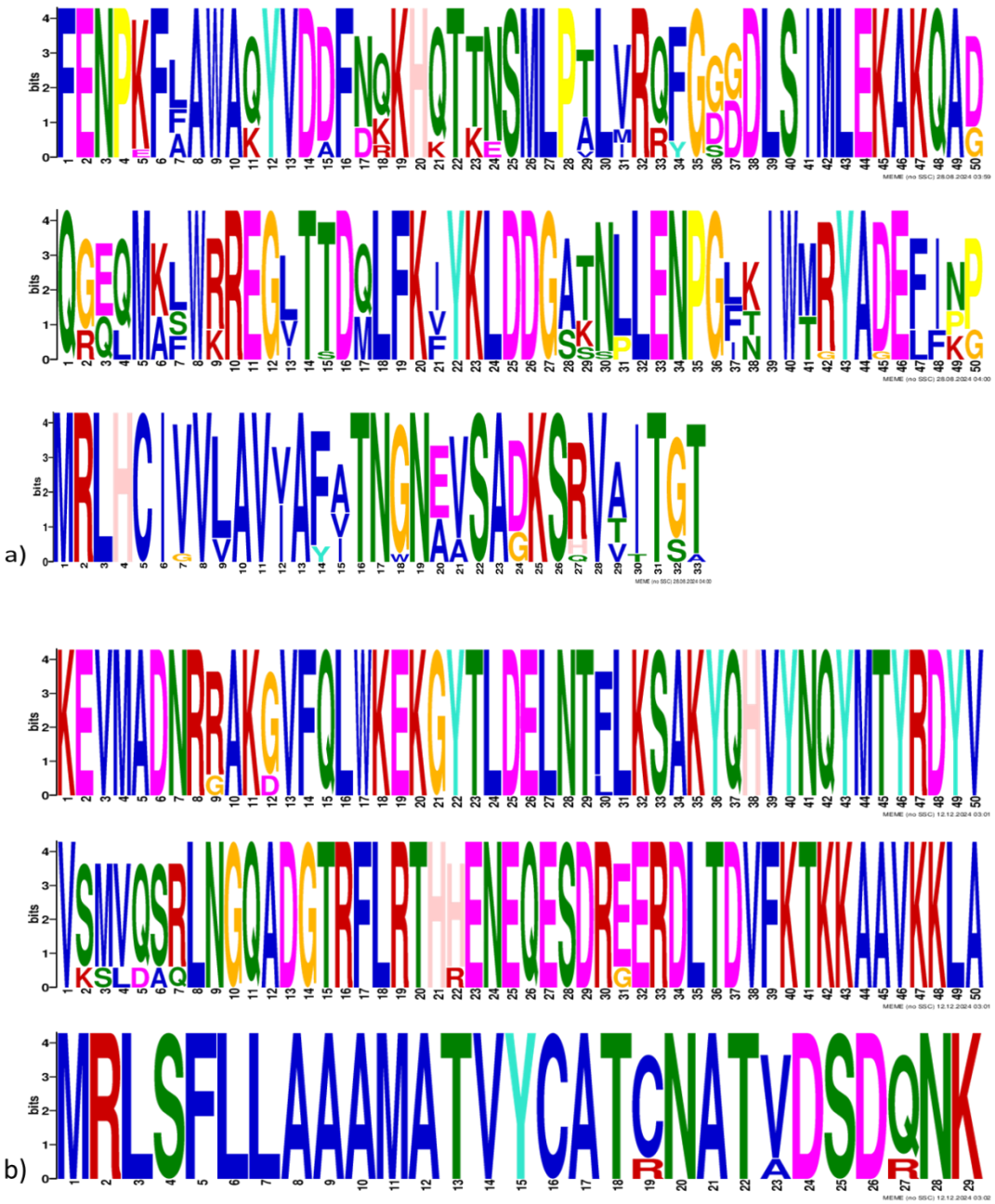
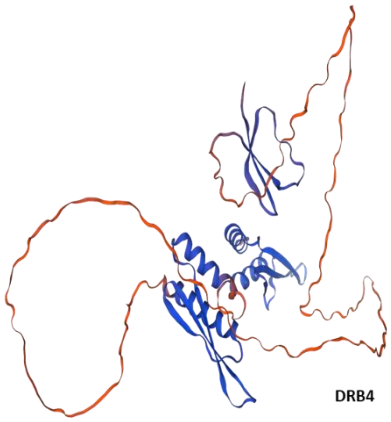
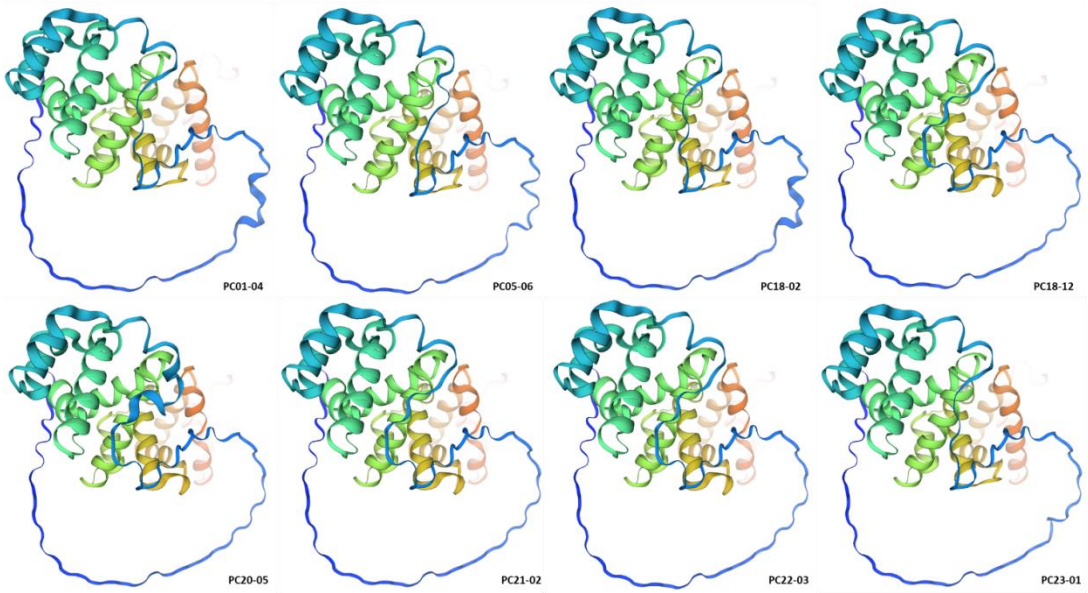


Fig. 7.8. Sequence logo of the identified motifs. a) RxLR29; b) RxLR132

7.3.3. Protein-protein interaction

Two of the highly expressed genes i.e., RxLR29 and RxLR132 during the infection were selected for the protein-protein interaction studies. To study the interaction between the effector RxLR29 and the RNA binding protein DRB4 and RxLR132 and CMPG1, structure prediction was performed using homology modeling approach. The homology model of the proteins predicted by SWISS-MODEL was depicted in Figures 7.9 & 7.10, which illustrates the presence of alpha helices and lengthy loops.





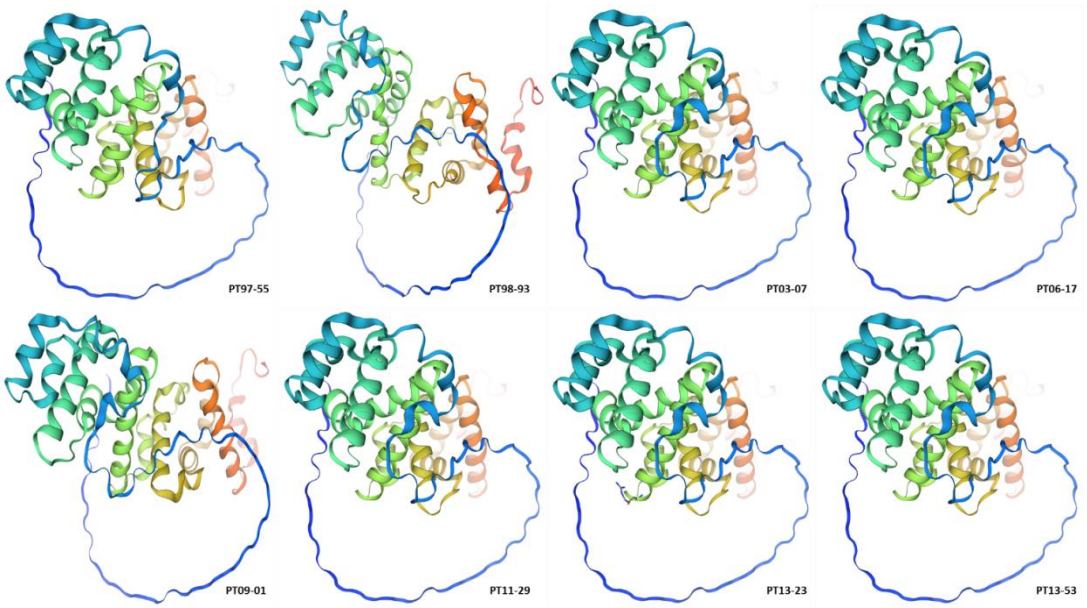


Fig. 7.9. Homology model of protein 3D structure of effector RxLR29 and its interacting protein DRB4

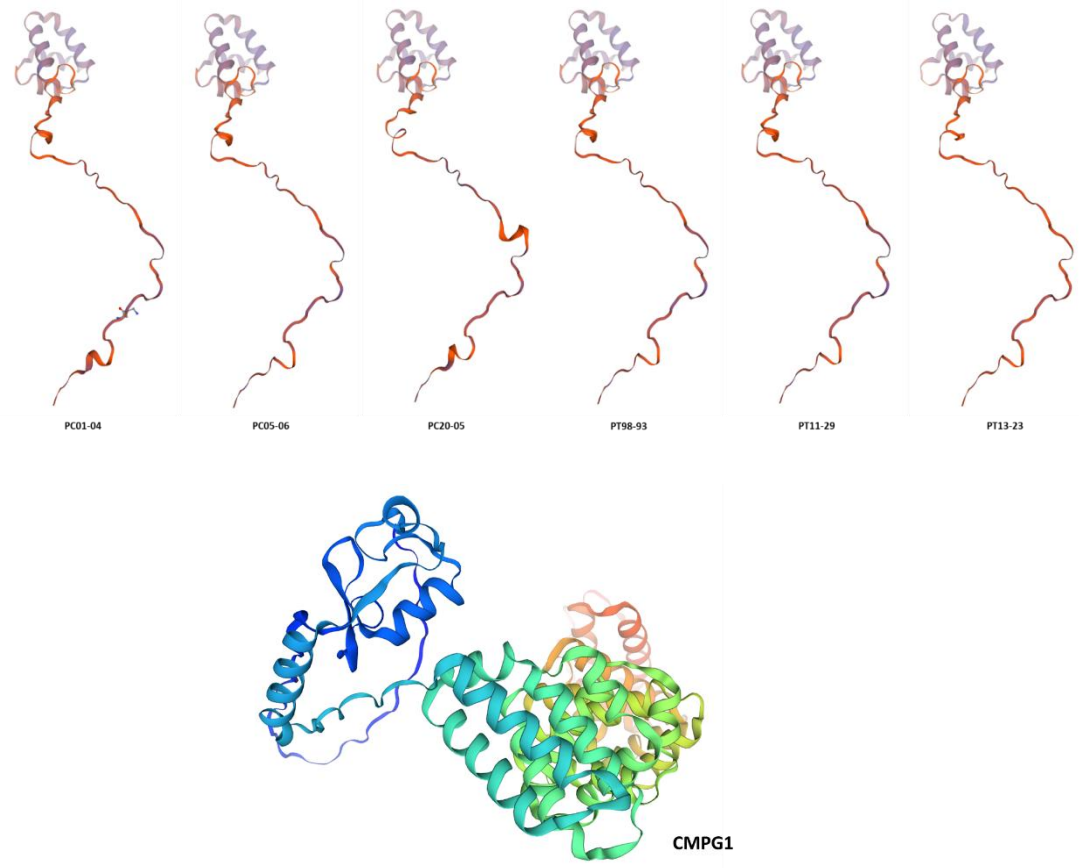


Fig. 7.10 Homology model of protein 3D structure of effector RxLR132 and its interacting protein CMPG1

The stereochemistry and the structural integrity were assessed by executing MolProbity in SWISS-MODEL (Table 7.3). Based on the torsional angles, the possible conformation of the amino acid residues was represented in the Ramachandran plot (Fig. 7.11, 7.12), where it can be seen that the majority of the residues fall into quadrants two and three, while a few residues can be spotted in the first quadrant. In the fourth quadrant, which is the unfavourable area due to the steric hindrance, it can be seen that one to two amino acid residues RxLR29 protein of very few isolates fall here and in RxLR132 (Table 7.4), two of the isolates have one amino acid residue each in the fourth quadrant. The overall MolProbity score of the RxLR29 protein is in the range of 1.21 to 1.95, indicates that predicted structures are in accurate conformations and that of RxLR132, the range was slightly greater i.e. 2.07 to 2.33, which are in the accepted range, also in case of DRB4, the MolProbity score is 2.07 shows that it is a medium-resolution structure with moderate issues whereas, for CMPG1 the score is 1.32, which is the accurate range. Clash score for the RxLR29 and DRB4 protein structure is within the range of 0.22 and 4.06, and for RxLR132, the range of clash score is from 4.92 to 5.5 and for CMPG1, the value is 2.09, which shows that the atomic clashes were very few and atoms are positioned in favourable conformations. Ramachandran favored region percentage shows that the RxLR29 protein of isolates PC01-04, PC05-06, PC18-02, PC23-01, PT03-07, PT06-17, PT09-01, PT11-29, PT13-23, PT13-53 and CMPG1 lies within the expected standards and for rest of the isolates PC18-12, PC20-05, PC21-02, PC22-03, PT97-55, PT98-93, DRB4 and RXLR132 of all isolates suggests that some residues may have unusual conformations. Rotamer outliers suggest a suboptimal side-chain orientation for RxLR132 of isolates except PC20-05 and DRB4. The observed values for the rest of the individual parameters, like C-beta deviations, bad bonds and angles, cis non-Proline, except for RxLR29 of PT06-17 and RxLR132 of PC20-05 and twisted non-Proline of RxLR29 isolates were slightly greater than the optimum range. However, the overall MolProbity score was within the accurate geometry.

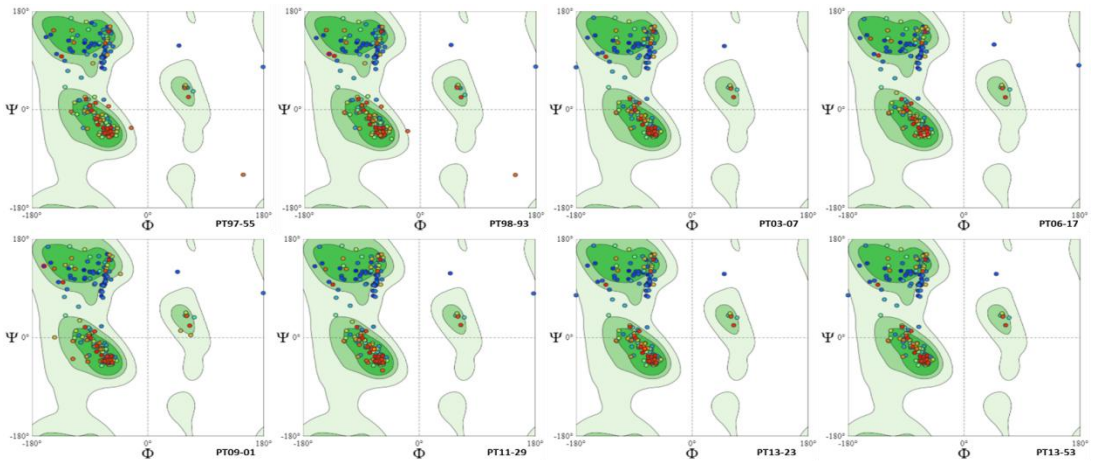


Fig. 7.11. Ramachandran plot of the predicted protein structures of RxLR29

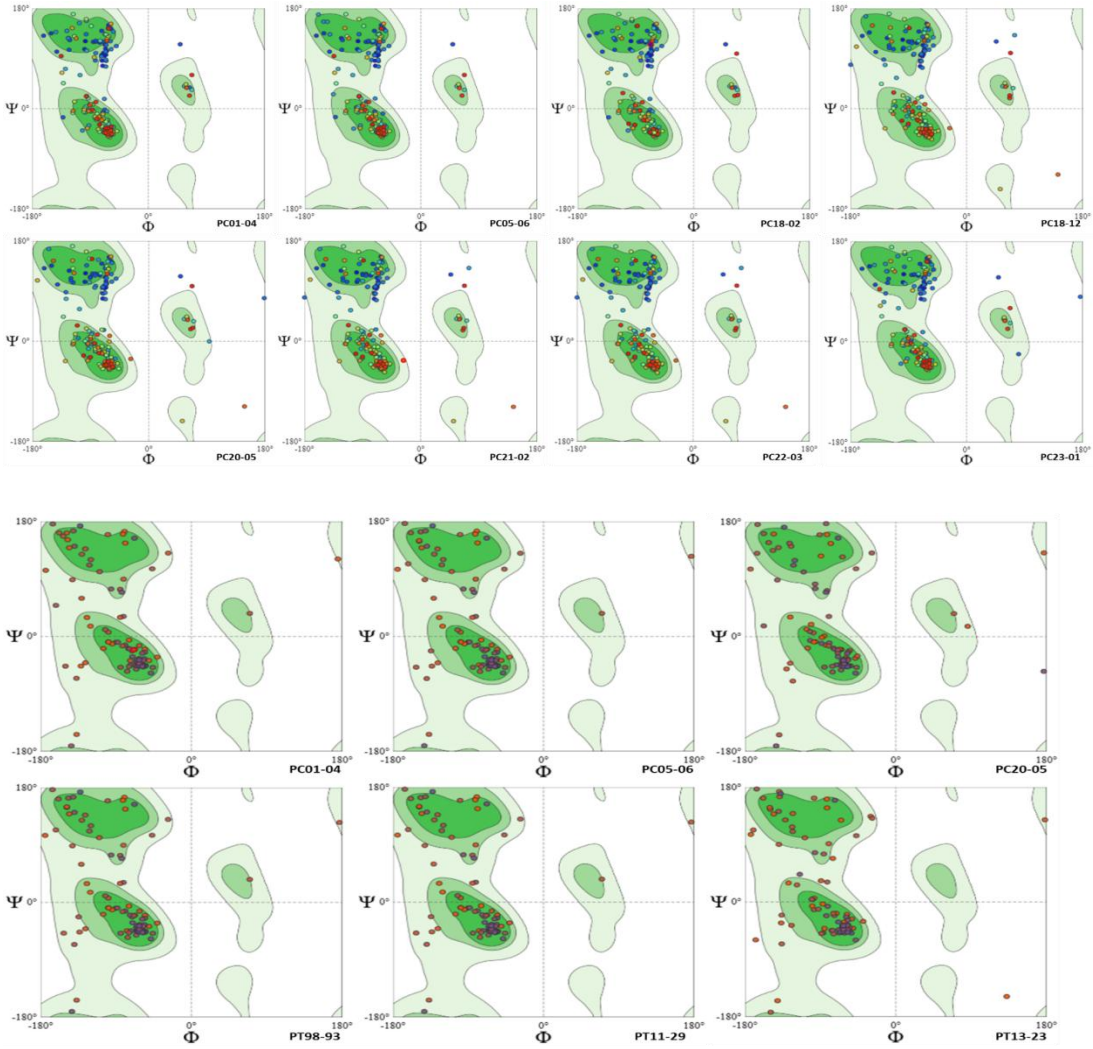


Fig. 7.12. Ramachandran plot of the predicted protein structures of RxLR132

Table 7.3. Evaluated parameters that validate the generated protein structure of RxLR29

Isolate	MolProbity Score	Clash score	*R favoured (%)	*R outliers (%)	*Ro outliers (%)	C-Beta deviations	Bad bonds	Bad angles	Cis Non-Proline	Twisted Non-Proline
PC01-04	1.93	4.04	91.27	0.73	2.03	1	3/2245	17/3024	-	3/268
PC05-06	1.93	3.82	90.91	0.36	2.03	1	2/2244	16/3025	-	3/268
PC18-02	1.94	4.06	91.24	1.09	2.04	1	3/2234	17/3009	-	3/267
PC18-12	1.51	0.67	86.72	2.21	1.65	5	0/2251	20/3035	-	3/263
PC20-05	1.95	3.08	87.27	2.55	2.03	5	2/2286	25/3085	-	3/266
PC21-02	1.51	0.67	86.72	2.58	1.66	5	0/2247	19/3030	-	3/263
PC22-03	1.51	0.67	86.72	2.58	1.66	5	0/2246	18/3028	-	3/263
PC23-01	1.96	4.05	90.55	1.45	2.05	1	3/2241	17/3018	-	3/268
PT97-55	1.94	3.58	89.74	1.83	2.07	1	0/2265	20/3056	1/266	3/266
PT98-93	1.78	2.69	89.74	1.83	1.67	2	0/2253	20/3035	1/266	3/266
PT03-07	1.21	0.22	92.50	1.07	1.63	0	0/2279	12/3076	1/271	3/271
PT06-17	1.44	0.87	91.10	1.78	1.61	0	0/2309	12/3114	2/272	3/272
PT09-01	1.33	0.66	90.04	1.42	1.22	0	0/2300	18/3101	1/272	3/272
PT11-29	1.47	1.53	91.10	1.78	1.21	0	0/2309	15/3113	2/272	3/272
PT13-23	1.23	0.22	92.14	1.07	1.63	1	0/2281	12/3078	1/271	3/271
PT13-53	1.21	0.22	92.50	1.07	1.62	0	0/2286	12/3085	1/271	3/271
DRB4	2.07	0.61	78.29	14.98	6.41	28	2/2567	82/3492	4/301	4/301

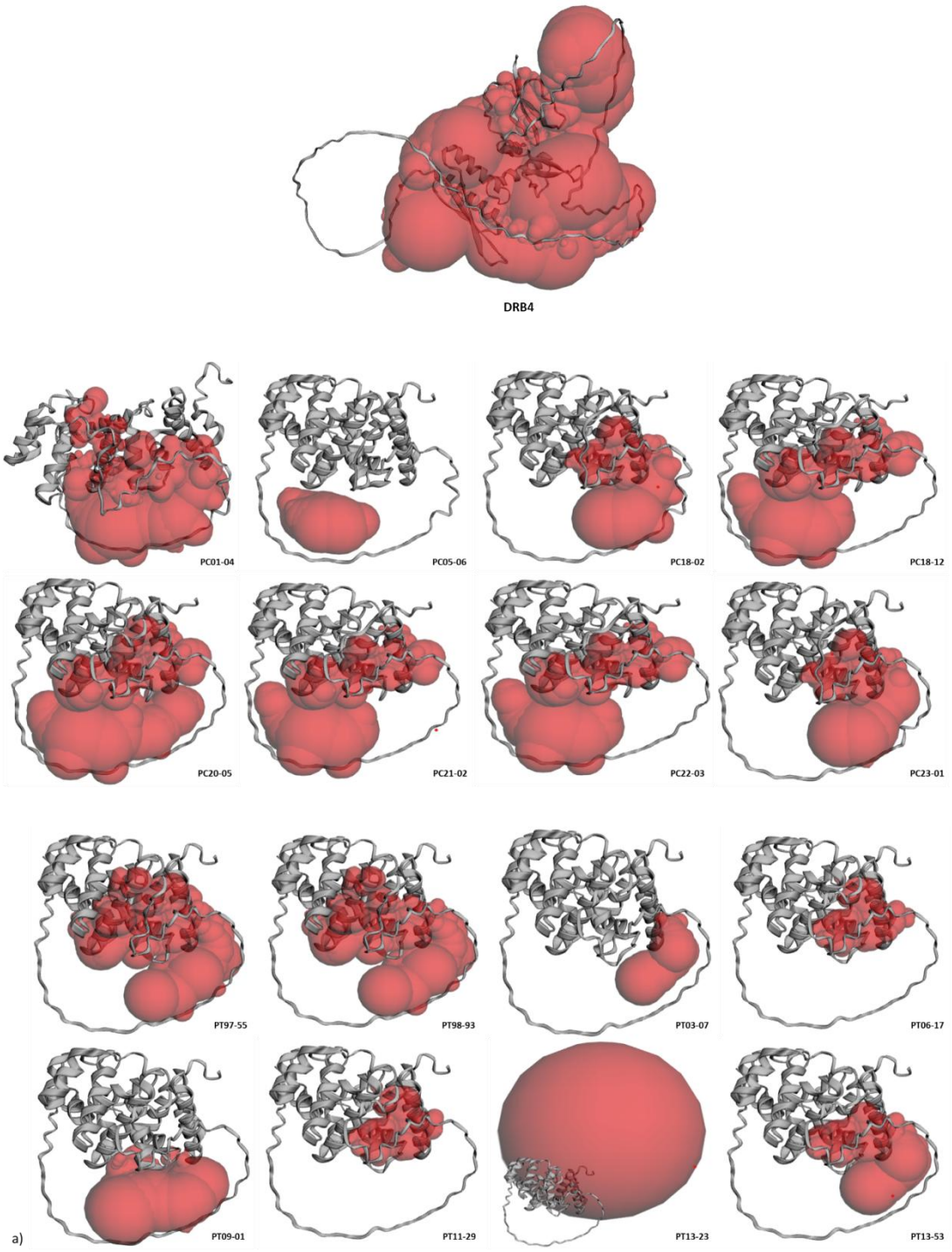
*R-Ramachandran, *Ro-Rotamer

Table 7.4. Evaluated parameters that validate the generated protein structure of RxLR132

Isolate	MolProbity Score	Clash score	*R favoured (%)	*R outliers (%)	*Ro outliers (%)	C-Beta deviations	Bad bonds	Bad angles	Cis Non-Proline	Twisted Non-Proline
PC01-04	2.24	4.92	87.27	1.82	3.03	1	0/938	8/1257	-	-
PC05-06	2.26	4.94	86.36	1.82	3.03	1	1/938	9/1258	-	-
PC20-05	2.07	4.84	88.50	0.88	2.00	2	0/957	10/1282	1/114	-
PT98-93	2.26	4.94	86.36	1.82	3.03	1	1/938	9/1258	-	-
PT11-29	2.26	4.94	86.36	1.82	3.03	1	1/938	9/1258	-	-
PT13-23	2.33	5.50	84.55	5.45	3.06	2	0/932	7/1250	-	-
CMPG1	1.32	2.09	95.98	0.67	1.22	3	0/3550	17/4786	1/436	2/436

*R-Ramachandran, *Ro-Rotamer

The CastP generated three-dimensional protein structure highlights the active sites or the surface available for the incoming protein to interact (Fig. 7.13). The details of active sites were used to define the active residues in HADDOCK 2.4 to obtain the accurate interactions.



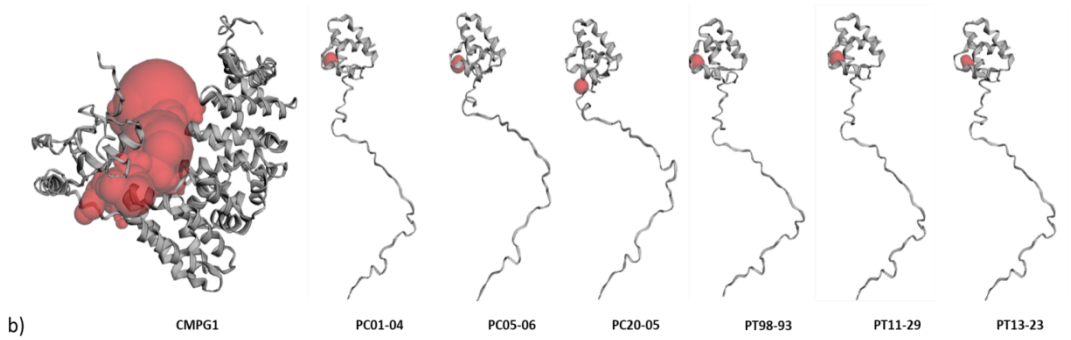
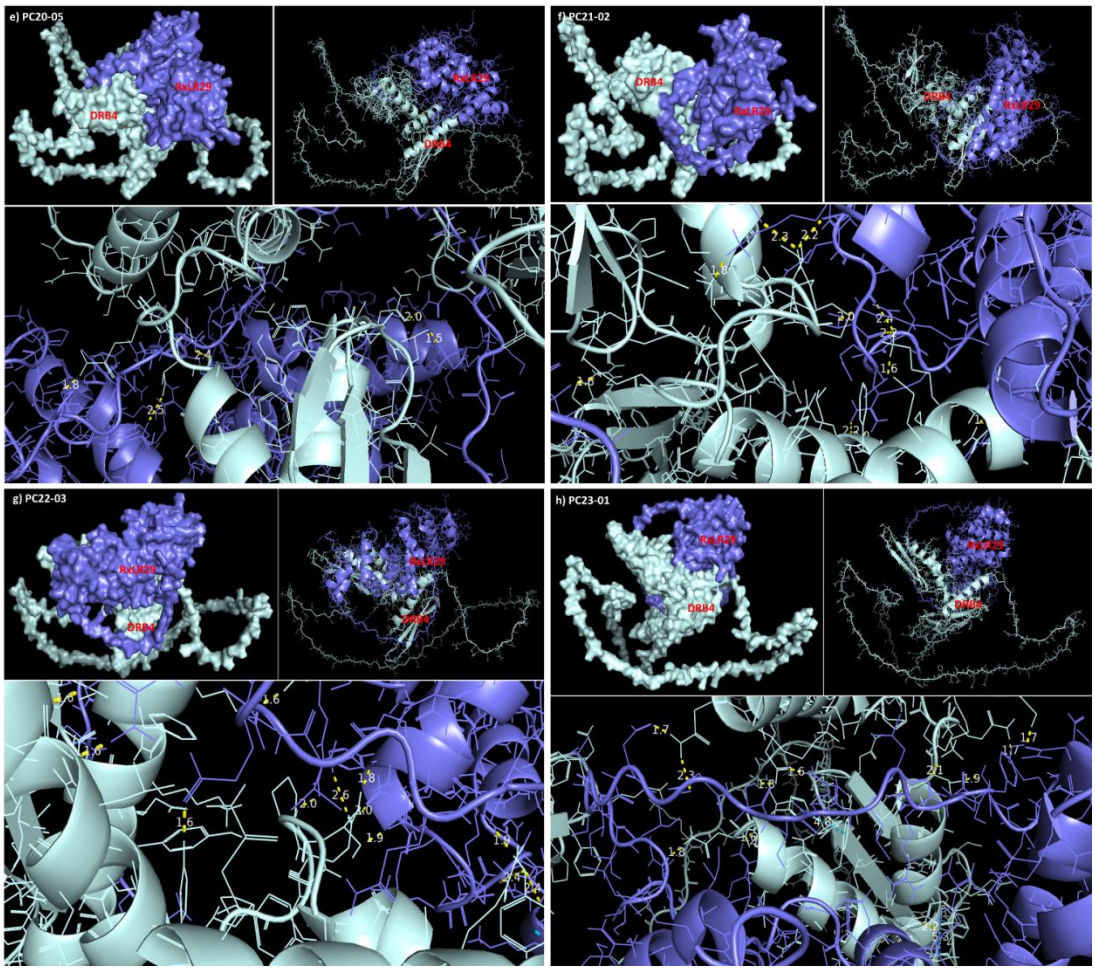


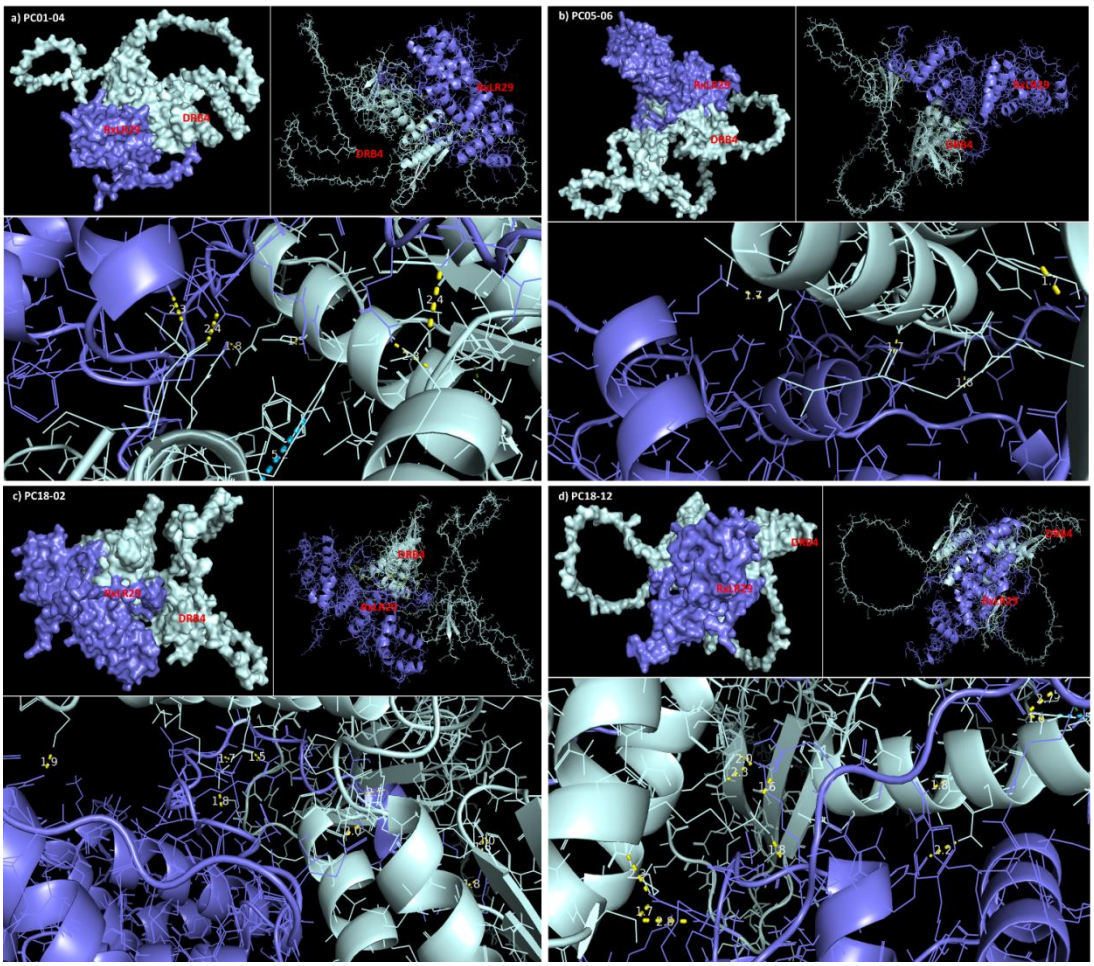
Fig. 7.13. Three-dimensional view of protein structures, the red colour shows the substrate binding sites, the main protein structure or the active site is represented using grey colour. a) PcLR29; b) RxLR132

The interaction between RxLR29 and DRB4 was resolved using HADDOCK 2.4. Based on the structural compatibility and binding affinity, the most favoured model is selected and visualized with PyMOL, Version 3.0 (Figs. 7.14, 7.15). In the diagram, the effector protein RxLR29 is represented in slate blue and DRB4 protein in a palecyan tint, the effector protein RxLR132 in magenta and CMPG1 in aquamarine tint. The surface diagram shows exactly the site of RxLR29 and DRB4 interaction. The cartoon representation brings a more transparent view, which highlights the secondary structures and the side chains. The zoomed view of the cartoon figure clearly shows the polar contacts labelled using a yellow dashed line, which specifies the non-covalent interactions like ionic interactions or hydrogen bonding that are fundamental in stabilizing interactions. The DRB4 protein structure displayed four alpha helices and three anti-parallel beta sheets and the structure of RxLR29 protein of all the isolates has 12 alpha helices and that of PT09-01, PT06-17 and PT11-29 has 13 alpha helices. The alpha helices were connected with lengthy prominent loops. In RxLR132 protein of the isolates has 5 alpha helices and 1 incipient helix and the structure of CMPG1 has 21 alpha helices, 1 incipient helix and 2 anti-parallel beta sheets.

Various matrices were used for the evaluation of protein-protein interaction (Tables 7.5, 7.6). The HADDOCK score, which is the combination of Van der Waals, restraint violation energy, electrostatic and desolvation energy is the overall score to rank the interaction, the more negative value is most favoured. In RxLR29, the

isolates PC05-06, PC18-02, PC23-01, PT03-07, PT06-17, PT09-01, PT11-29, PT13-23 and for all the isolates of RxLR132 the HADDOCK scores were within the favoured range. The Z-score explains the extent of deviation of HADDOCK score from the mean of a particular cluster to the average of all the clusters, and the Z-scores for all isolates indicate that the docking model is reliable and energetically favourable.




a) PC01-04
b) PC05-06
c) PC18-02
d) PC18-12

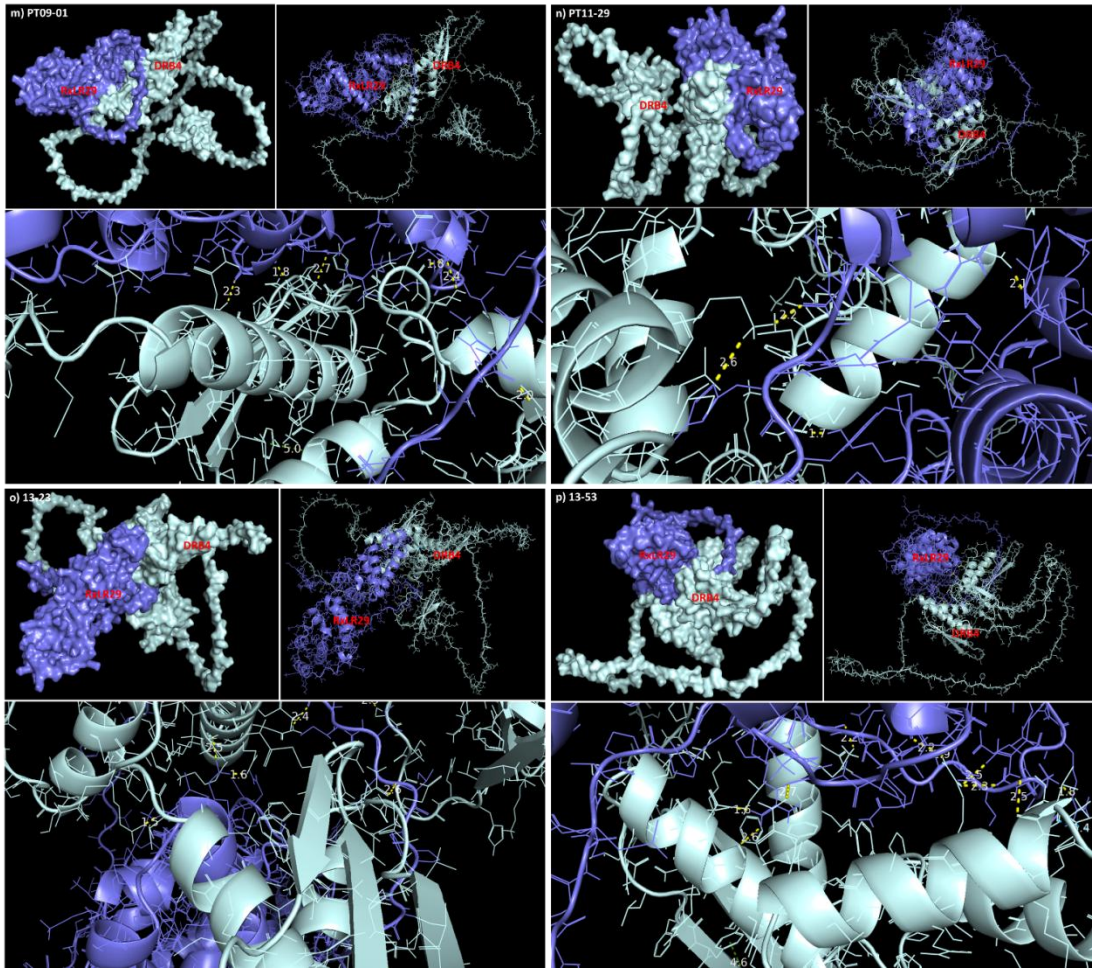
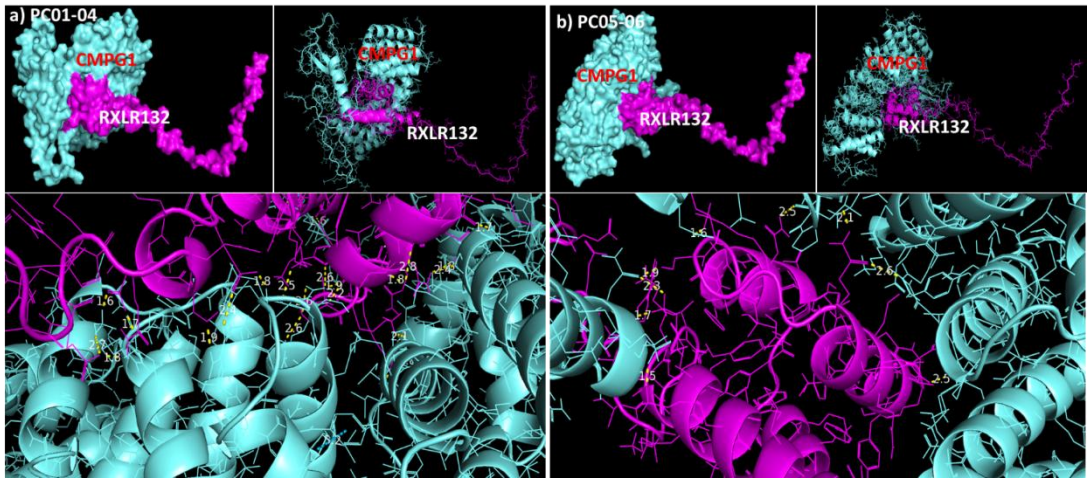


Fig. 7.14. Protein-protein interaction between RxLR29 and DRB4. Top left shows the 3D surface image, top right displays the cartoon model and the bottom image is the closer view showing the hydrogen bonds between RxLR29 and DRB4 proteins represented by yellow dotted lines



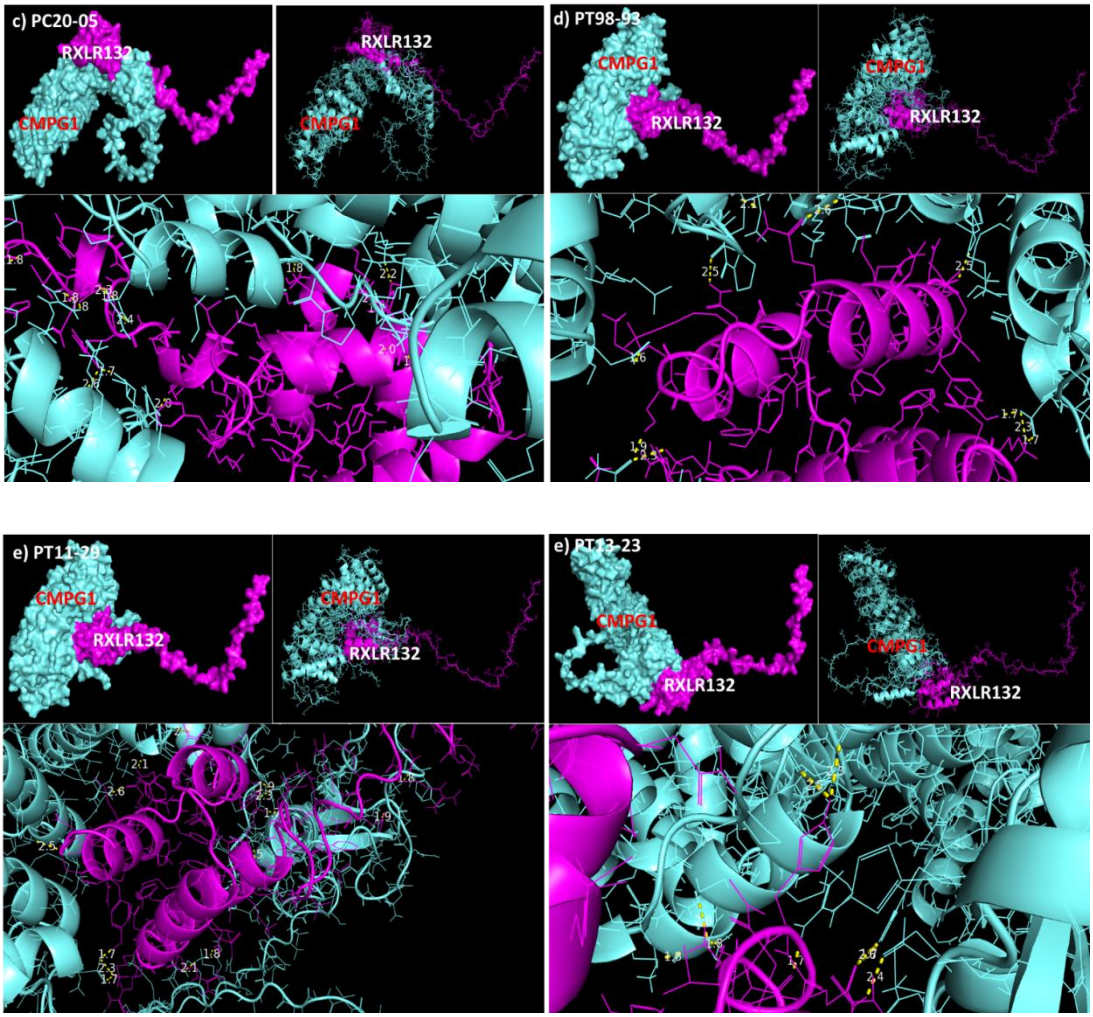


Fig. 7.15. Protein-protein interaction between RxLR132 and CMPG1. The top left shows the 3D surface image, the top right displays the cartoon model and the bottom image is a closer view showing the hydrogen bonds between RxLR132 and CMPG1 proteins represented by yellow dotted lines

Table 7.5. Validation of interaction between RxLR29 and DRB4 based on cluster analysis and energy terms

Isolates	Haddock score	Cluster size	RMSD	Van der Waals energy	Electrostatic energy	Desolvation energy	Restraints violation energy	Buried surface area	Z-score
01-04	7.8.0±18.0	9	24.8±0.4	-87.2±11.4	-453.8±72.7	8.4±2.9	1774.1±142.3	3942.8±-193.9	-2.5
05-06	-76.7±7.9	6	28.5±0.1	-50.6±5.2	-242.7±11.2	-7.3±2.5	297.2±37.8	2199.5±-167.1	-1.2
18-02	-68.5±14.4	8	26.2±0.2	-66.8±6.8	-448.4±33.1	13.1±3.3	749.3±151.7	3416.2±-57.2	-2.1
18-12	-7.8±19.8	4	28.9±0.1	-85.5±11.1	-320.9±61.8	22.8±5.8	1190.5±81.3	3514.3±-335.2	-1.6
20-05	4.6±22.3	6	1.1±0.7	-90.5±17.4	-310.7±62.9	2.0±10.8	1552.8±207.7	3717.6±280.3	-1.8
21-02	-0.2±11.4	6	1.0±0.6	-76.0±8.5	-530.3±23.7	40.1±1.5	1418.2±90.6	3525.2±187.5	-1.5
22-03	17.2±16.5	7	2.3±0.3	-54.4±8.9	-405.1±48.6	27.9±-4.9	1247.0±176.0	2864.0±190.6	-2.0
23-01	-60.3±9.6	11	2.5±0.7	-69.3±7.3	-372.8±86.8	10.3±6.1	732.5±34.6	3175.3±264.6	-2.2
97-55	3.9±10.9	8	25.2±0.2	-63.5±8.8	-363.7±27.9	17.7±3.5	1224.2±45.1	2846.2±154.6	-1.8
98-93	-26.0±9.8	41	29.2±0.1	-76.9±11.1	-400.3±48.6	21.3±2.0	1097.3±127.4	3201.1±319.4	-1.8
03-07	-83.5±6.1	11	18.4±0.3	-64.8±5.0	-143.5±51.5	-12.4±4.1	224.0±60.5	2144.5±111.8	-2.2
06-17	-75.6±11.4	8	0.8±0.4	-74.1±8.5	-267.5±19.2	-8.2±2.7	602.2±49.4	2832.8±178.7	-2.1
09-01	-83.7±9.6	54	1.2±0.7	-78.3±12.2	-287.8±26.8	13.4±3.5	387.1±32.0	3061.6±181.5	-1.6
11-29	-67.6±4.5	11	28.9±0.1	-73.3±9.9	-305.2±31.9	-5.1±1.6	718.5±59	2942.9±152.7	-1.5
13-23	-96.1±5.4	117	2.4±0.7	-47.0±10.3	-257.6±46.2	-9.6±5.6	120.3±61.2	2180.6±220.5	-1.6
13-53	-32.3±28.7	5	1.9±1.5	-56.9±14.5	-249.2±39.9	2.6±6.2	718.7±110.5	2622.8±246.2	-1.6

*RMSD-RMSD from overall lowest energy structure

Table 7.6. Validation of interaction between RxLR132 and CMPG1 based on cluster analysis and energy terms

Isolates	Haddock score	Cluster size	RMSD	Van der Waals energy	Electrostatic energy	Desolvation energy	Restraints violation energy	Buried surface area	Z- score
01-04	-83.7 ±17.7	5	0.8 ± 0.4	-60.5 ±12.0	-727.6 ± 47.8	49.3±4.0	729.3±77.7	3308.9±166.0	-2.0
05-06	-53.9±23.6	12	3.5±2.7	-57.8±7.1	-571.5±58.7	23.1± 4.9	951.4±114.2	2755.4±229.8	-1.6
20-05	-45.4±3.8	5	26.3±0.4	-54.3±7.1	-506.6±12.3	13.6±3.0	966.1±48.8	2445.4±286.2	-1.9
98-93	-53.8 ±23.7	12	3.5±2.7	-57.8±7.1	-571.2±58.6	23.1±4.8	951.5±114.3	2753.7±230.5	-1.6
11-29	-53.9±23.6	12	3.5±2.7	-57.8±7.1	-571.5±58.7	23.1±4.9	951.4±114.2	2755.4±229.8	-2.0
13-23	-49.4±17.9	25	16.7±0.4	-63.5±8.9	-289.1±10.1	1.2±2.0	706.5±82.7	2011.5±148.7	-1.6

*RMSD-RMSD from overall lowest energy structure

7.4. Discussion

One of the advanced mechanisms adopted by the pathogen to suppress the immune response of the host plant and to establish infection is via the secretion of effector molecule, which mainly targets the physiological process or the immune components. The RxLR effector molecules are the prominent class of effectors which are widely studied in *P. capsici* (Li *et al.,* 2019b). However, no reports were available on the study of the effector molecules in *P. tropicalis.* According to Li *et al*. (2022), only a few of the RxLR regions are conserved among species, and sixteen of the RxLRs which are homologous were found in *P. ramorum, P. infestans* and *P. sojae*.

Following the infection of *P. capsici,* all the analyzed RxLR genes were up-regulated, and the expression of RxLR132 was the greatest. A study involving RxLR132 of *Phytophthora* showed that the upregulation of VPS genes in *Nicotiana benthamiana* is essential for the progress of the infection (Lu *et al.,* 2020). A five-fold increase in the expression of RxLR29 was observed following the infection in black pepper. However, RxLR29 gene of *P. agathidicida* was not expressed in *Agathis australis* during infection (Cox *et al.,* 2022). RxLR48 was expressed only 1.6-fold during infection in black pepper, and it has a role in suppressing the Salicylic acid-mediated immune response by associating with NPR1 (non-expressor of pathogenesis-related-1) and suppresses (PTI) pattern-triggered immunity in *N. benthamiana* (Li *et al.,* 2019b). A 3-fold increase in the expression of RxLR103 was observed, and it acts by targeting EDS1 (enhanced disease susceptibility1) of the host and inhibit the formation of EDS1/PAD4 (Phytoalexin Deficient4) complex, which ultimately hinders the salicylic acid-mediated immune response (Li *et al.,* 2017). RxLR207 expressed 4-fold during *Phytophthora* infection in black pepper and it acts by interfering in the ROS-mediated immune response of the host by degrading the regulators such as BPA1 (binding partner of ACD11) and BPLs (BPA1-Like proteins) in *Arabidopsis* (Li *et al.,* 2019a).

Here, the RxLR29 identified from *P. capsici* and *P. tropicalis* illustrates the typical structure of RxLR effector in a way that it has a signal peptide domain towards the N-terminal domain, RxLR-DEER motif (Yin *et al.,* 2017) and a variable effector domain towards the C-terminal (Rojas-Esteves *et al.,* 2020). Earlier studies reported

that the RxLR29 sequence was highly variable among *Hyaloperonospora arabidopsidis* isolates, which acts by hindering the callose deposition by the pathogenic bacteria in the host (Cabral *et al.,* 2011). Wawra *et al.,* (2017) showed that in *P. infestans,* the RxLR sequence of AVR3a gets cleaved and the N-terminal undergoes acetylation before translocating into the host cell. However, the DEER motif was absent in RxLR132, which plays a critical role in the effector translocation into the host cell (Bouwmeester *et al.,* 2011). Three consensus domains were identified at the highly variable effector domain, suggesting that these motifs were functionally essential for the infection process. Hence, it holds evolutionary pressure to maintain the function (Itoh *et al.,* 2007; Vaschetto, 2022). These regions also contribute to the improved stability of protein and its biological activity (Sternke *et al.,* 2019).

P. capsici is a pathogen with a broad host range that includes model plants such as *Nicotiana benthamiana* and *Arabidopsis thaliana* (Li *et al.,* 2019b; Naveed *et al.,* 2020). To study the interaction between RxLR29 and DRB4 proteins, the DRB4 protein from *Arabidopsis thaliana* is selected since the DRB4 sequence was not available so far for the black pepper host plant. Similarly, to study the RxLR132 and CMPG1, the CMPG1 sequence of *Solanum lycopersicum* was retrieved.

The present study identified the possibilities of interaction between RxLR29 and DRB4, and also RxLR132 with CMPG1. Wang *et al*. (2018) and Gilroy *et al.* (2011) have reported the role of AVR3a in stabilizing U-box E3 ligase and thereby inhibiting the INF1-triggered cell death in *N. benthamiana*. *Phytophthora* suppressor of RNA silencing 2 (PSR2) of *P. sojae* acts by suppressing the RNA silencing in the host plant, thus compromising its immune response in Soybean and *Arabidopsis thaliana* and these sequences are conserved across *Phytophthora* species (Wood *et al.,* 2020). Using the HADDOCK score, Suganthi *et al*. (2024) validated the interaction between GAPDH and chitinase. The antifungal potential of Bacillus lipopeptides against *P. infestans* effector protein was confirmed in silico and validated using HADDOCK (Kumbar *et al.,* 2021). Further, the virulence of these isolates against the susceptible variety of black pepper i.e., Sreekara (Chapter 5), was compared with the protein-protein interaction scores. Though the pattern was not proportional, most virulent isolates showed good interaction scores with few exceptions i.e., in the case of PT13-35, which showed high virulence but its

interaction scores were poor for RxLR29 and a moderate score in RxLR132 interaction. The isolates PT03-07 and PC01-04 were less virulent, but the former had a good interaction score in RxLR29 interaction and was not amplified for RxLR132. The latter gave a poor interaction score in RxLR29 but a better interaction score in case RxLR132. The studies which correlate the virulence and the protein-protein interaction between virulence and avirulence proteins of the host and the pathogen respectively, are limited. However, there are reports suggesting a direct relation between virulence, for instance, *P. infestans* infection in *N. benthamiana* (Yin *et al.,* 2017) and *P. parasitica* infection in *A. thaliana* and *N. benthamiana* (Huang *et al.,* 2018).

7.5. Conclusion

Phytophthora is a group of fungus-like oomycete pathogens which is a common threat to a broad range of commercially important crops. *P. capsici and P. tropicalis* are often found associated with the foot rot disease of black pepper. During the infection, it was found that these genes are upregulated, suggesting their unavoidable role in the progression of the infection process. The analysis of gene sequences of RxLR29 and RxLR132 effector molecules in the present study showed that variation exists in both gene sequences among the selected isolates. Variations are mainly of nonsynonymous substitutions, insertions and deletions. The prediction of the RxLR motif and signal peptide region was performed, and the results showed that there exists a difference in the length of the signal peptide sequence of both genes. Homology modeling and protein-protein interaction studies revealed that the effector proteins of *P. capsici* and *P. tropicalis* such as RxLR29 and RxLR132 interact with DRB4 and CMPG1 proteins of the host, hence hindering the immune response and making it more susceptible. Future line of research can be focused on finding the homologue of DRB4 and CMPG1 in black pepper and its interaction with the effectors experimentally, hence resolving its mode of action in the black pepper pathogenesis.

CHAPTER 8
SUMMARY

Proper identification of the causative agent of a disease and timely action taken to restrict its growth and establishment is highly critical in its management. The foot rot disease of black pepper caused by *P. capsici* and *P. tropicalis* is prevalent in major black pepper growing regions in India. Here we used PCR assay with species-specific primers to identify at the species level. It was observed that the number of both *P. capsici* and *P. tropicalis* isolates identified was almost equal. Other species were also identified such as *P. nicotianae*, *P. meadii* and *P. palmivora,* which were identified in very less numbers, which suggests that *P. capsici* and *P. tropicalis* were the two major species associated with the disease.

The information about the occurrence of mating type gives an idea on the possible reason behind the pathogen population i.e., clonal or genetically variable. The mating type of the *Phytophthora* isolates in the present study was identified as A1 using the pairing test with the reference isolate of known mating type. For the quick identification of the mating types, we also attempted to develop a mating type-specific marker and the activity encountered a temporary setback due to the unavailability of A2 mating type of *P. capsici*/*P. tropicalis* from black pepper host. Further, the validation of an already reported primer PCAP1 (5'-ACGAGTACGAGTGCTTGGT-3') and PCAP2 (5'-TGAGTCTCGAGAC AGAGAG-3') which could distinguish the mating type in *P. capsici* of *Capsicum annuum* was proved to be unreliable to differentiate mating types of the isolates infecting black pepper.

The inter/intra species variation ultimately gets reflected in its genome size. The genome size of *P. capsici* and *P. tropicalis* isolates was studied using one of the widely accepted techniques, flow cytometry. The observations show that the genome size is variable between and among the species of the isolates indicating a genetically variable isolates within the population. The correlation analysis between

the morphological characters such as mycelial growth and zoospore production and virulence and fungicide resistance shows no significant positive correlation.

The genetic variation among the population was further evaluated using dominant markers like RAMS and REP-PCR, which were efficient in distinguishing the variation even within the species. The analyses revealed the occurrence of variability among the isolates and were able to distinguish them at the species level and REP-PCR was found to be slightly more precise. The morphological characters, virulence pattern, geographical origin and genetic variability of the pathogen were found to be independent, indicating a widespread dispersal of the pathogen. The cross-infectivity *in vitro* analysis revealed the ability of black pepper isolates to infect other crops like chilli, tomato, pumpkin, nutmeg, cucumber and a few isolates infected cardamom.

To identify the changes within a gene that are resultant of evolutionary forces were analyzed by studying the mitochondrial and nuclear genes. The demographic analysis revealed a bottleneck event which reduced the genetic diversity but the balancing selection restored the variation after the bottleneck event followed by a population expansion. The phylogenetic analysis showed the close relationship of Indian black pepper *Phytophthora* isolates with that of the United States population, indicating its widespread dispersal.

Further, to have a better understanding on the pathogen virulence, ten different RxLR effectors were studied for the selected isolates. Based on the expression analysis, two of the highly expressed RxLRs i.e., RxLR29 and RxLR132 during *P. capsici* infection in black pepper were chosen for the rest of the analysis. The RxLR regions too showed variability among the isolates. Protein-protein interaction of the selected RxLRs was studied with their interacting protein homologue and found that biologically stable interaction exists between RxLR29 and DRB4 and RxLR132 and CMPG1.

CHAPTER 9
RECOMMENDATIONS

Foot rot caused by *Phytophthora* is among the dreadful diseases of black pepper which drastically affects its cultivation. Bridging the knowledge gap in the population biology of *Phytophthora* aids in the effective management of the disease for the betterment of plant health. In the present study, *Phytophthora* species infecting black pepper were identified and its distribution and mating type was also studied. Further, frequent monitoring for the disease occurrence and presence of A2 mating type is highly recommended to check black pepper cultivation from the rampant effect of the disease. The absence of A2 mating was one of the challenges faced during the development of mating-type specific marker and further attempts to develop mating-type specific marker can be made.

The study also focused on the flow cytometry analysis of *P. capsici* and *P. tropicalis* which gave us the basic knowledge on the genome content of the isolates. Besides, whole genome studies can give us accurate information on the genome size of the isolates and the genes associated with the phenotypic variability of the pathogen.

Genetic diversity of *Phytophthora* isolates was studied using, RAMS and REP-PCR techniques, which gave us an idea regarding the composition of variation that exists among the Indian population. Though there exist several driving forces behind these variations, the exact reason can be explored further.

Haplotype analysis revealed the genetic variations of *P. capsici* and *P. tropicalis* isolates concerning the mitochondrial and nuclear genes. The bottleneck event which resulted in the loss of genetic variation can be investigated and further study recommendations can include tracing back the evolutionary relations and its migration which resulted in its occurrence in India.

The *in-silico* studies on the highly expressed RxLR genes during infection unveiled the possible interaction of these RxLRs with their interacting protein homologue. Further, the mode of action of these effector molecules can be studied specifically

with its interacting protein counterpart in black pepper, which will give us a better understanding on its target and how it ultimately contributes to its virulence.

The study of the population biology of *Phytophthora* is vast, and the present study attempted to study the aforementioned aspects which opened door to explore more into the specific aspect of the research problem.

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APPENDIX

Media preparations

Carrot agar

Carrot	200g
Agar	15g
Sterile distilled water	up to 1000 mL

Fresh carrot was peeled and chopped into small pieces and ground well with 500 mL sterile distilled water using a blender. Carrot juice was collected by filtering using sterile muslin cloth and the volume was made up to 1000 mL. Agar was added and the mixture was boiled. The layer of foam was removed and was autoclaved at 121.6^{o}C for 30 min at 15 lbs.

Ribeiro's broth

Microelements

Sodium molybdate	41.1 mg
Zinc sulphate	87.8 mg
Copper sulphate	7.85 mg
$MnSO_4$	15.4 mg
Sodium borate	0.5 mg

Dissolve the above components in 100 mL distilled water.

Ferric chloride (stock solution)

EDTA	2.6 g
KOH	1.5 g
$FeCl_2$	50 mg

Dissolve the above components in 100 mL distilled water.

Thiamine (stock solution)

Thiamine HCl	10 mg

Dissolve the above component in 10 mL distilled water and sterilize by Millipore filtration.

Basal media

Glucose	4.5 g
L-Asparagine	0.1 g
KNO_3	0.15 g
KH_2PO_4	1.0 g
$MgSO_4$	0.5 g
$CaCl_2$	0.1 g
B-Sitosterol	30 mg (dissolved in 30 mL DMSO/Ethanol)

Dissolve the above components and make up the volume to 1000 mL.

1 mL each of microelement solution and Ferric chloride (stock solution) was added to the basal medium. pH was adjusted to 6.2 with 6 N KOH and were autoclaved at 121.6^{o}C for 30 min at 15 lbs. When the temperature drops to room temperature, 1 mL of the sterilized thiamine solution was added and used for inoculation.

Reagent preparations

TAE buffer, 50 X stock

Tris base	242.2 g
Glacial acetic acid	57.1 mL
0.5 M EDTA	100 mL

Dissolve the above components and make up the volume to 1000 mL

STE extraction buffer (50 mL)

1M Tris HCl	10 mL
5M NaCl	2.5 mL
0.5M EDTA	2.5 mL
10% SDS	2.5 mL

Dissolve the above components and make up the volume to 50 mL

Accession numbers of the sequences deposited in NCBI.

Isolate (EF-1α)	(Enolase)	(β-tubulin)	(TigA)	(Ura3)	(Nad1)	(Cox1)	(Cox2)	(Nad5)	(HSP90)
PC01-04OP432593	OP502881	OP502905	OP502929	OP502953	OP502977	OP503001	OP681087	OP604575	OP681111
PC02-20OP432594	OP502882	OP502906	OP502930	OP502954	OP502978	OP503002	OP681088	OP604576	OP681112
PC05-06OP432595	OP502883	OP502907	OP502931	OP502955	OP502979	OP503003	OP681089	OP604577	OP681113
PC06-12OP432596	OP502884	OP502908	OP502932	OP502956	OP502980	OP503004	OP681090	OP604578	OP681114
PC07-03OP432597	OP502885	OP502909	OP502933	OP502957	OP502981	OP503005	OP681091	OP604579	OP681115
PC10-02OP432598	OP502886	OP502910	OP502934	OP502958	OP502982	OP503006	OP681092	OP604580	OP681116
PC11-13OP432599	OP502887	OP502911	OP502935	OP502959	OP502983	OP503007	OP681093	OP604581	OP681117
PC11-16OP432600	OP502888	OP502912	OP502936	OP502960	OP502984	OP503008	OP681094	OP604582	OP681118
PC13-17OP432601	OP502889	OP502913	OP502937	OP502961	OP502985	OP503009	OP681095	OP604583	OP681119
PC13-42OP432602	OP502890	OP502914	OP502938	OP502962	OP502986	OP503010	OP681096	OP604584	OP681120
PC18-12OP432603	OP502891	OP502915	OP502939	OP502963	OP502987	OP503011	OP681097	OP604585	OP681121
PC20-05OP432604	OP502892	OP502916	OP502940	OP502964	OP502988	OP503012	OP681098	OP604586	OP681122
PT97-55OP432605	OP502893	OP502917	OP502941	OP502965	OP502989	OP503013	OP681099	OP604587	OP681123
PT98-93OP432606	OP502894	OP502918	OP502942	OP502966	OP502990	OP503014	OP681100	OP604588	OP681124
PT00-38OP432607	OP502895	OP502919	OP502943	OP502967	OP502991	OP503015	OP681101	OP604589	OP681125
PT01-20OP432608	OP502896	OP502920	OP502944	OP502968	OP502992	OP503016	OP681102	OP604590	OP681126
PT03-07OP432609	OP502897	OP502921	OP502945	OP502969	OP502993	OP503017	OP681103	OP604591	OP681127
PT06-17OP432610	OP502898	OP502922	OP502946	OP502970	OP502994	OP503018	OP681104	OP604592	OP681128
PT08-07OP432611	OP502899	OP502923	OP502947	OP502971	OP502995	OP503019	OP681105	OP604593	OP681129
PT09-01OP432612	OP502900	OP502924	OP502948	OP502972	OP502996	OP503020	OP681106	OP604594	OP681130
PT11-20OP432613	OP502901	OP502925	OP502949	OP502973	OP502997	OP503021	OP681107	OP604595	OP681131
PT11-29OP432614	OP502902	OP502926	OP502950	OP502974	OP502998	OP503022	OP681108	OP604596	OP681132
PT13-23OP432615	OP502903	OP502927	OP502951	OP502975	OP502999	OP503023	OP681109	OP604597	OP681133
PT13-53OP432616	OP502904	OP502928	OP502952	OP502976	OP503000	OP503024	OP681110	OP604598	OP681134

PUBLICATIONS AND PRESENTATIONS

Publications

→ Zumaila, F., Jeevalatha, A., & Biju, C. N. (2026). Haplotype studies and population structure analysis of the South Indian population of *Phytophthora* species infecting black pepper. *Fungal Biology*, *130*(1), 101693. https://doi.org/10.1016/j.funbio.2025.101693

→ Zumaila, F., Jeevalatha, A., & Biju, C. N. (2024). Genetic diversity, mating type and pathogenicity of two *Phytophthora* species infecting black pepper in India. *3 Biotech, 14*(1), 1. https://doi.org/10.1007/s13205-023-03843-1

→ Jeevalatha, A., Zumaila, F., Biju, C. N., & Punya, K. C. (2022). Duplex recombinase polymerase amplification assay for simultaneous detection of *Pythium* spp. and *Ralstonia pseudosolanacearum* from ginger rhizomes. *Crop Protection*, 161, 106057.

→ Amrutha, L. M., Suresh, K., Challa, G. K., Zumaila, F., George, P., & Praveena, R. (2024). Effect of plant protection chemicals on the survivability of *Phytophthora* spp. infecting black pepper (*Piper nigrum* L.). *Journal of Mycology and Plant Pathology, 54*(1), 1-11

→ Jeevalatha, A., Zumaila, F., Biju, C. N., & Peeran, M. F. (2022). Multiplex PCR assay for simultaneous detection of *Phytophthora*, *Pythium* and *Fusarium* associated with foot rot and yellowing diseases of black pepper. *Journal of Spices & Aromatic Crops, 31*(1).

Oral Presentations

→ Zumaila, F., Jeevalatha, A. and Biju, C. N. (2021). Evaluation of PCR markers for mating type determination in *Phytophthora* spp. infecting black pepper. "Symposium on Sustainable Plant Health Management Amidst Covid Pandemic: Challenges and Strategies", Indian Phytopathological Society (South Zone Chapter), 1-3 December 2021, ICAR- Central Plantation Crops Research Institute, Kasaragod, Kerala.

Poster presentations

→ Zumaila, F., Jeevalatha, A. and Biju, C. N. 2022. Haplotype analysis indicates genetic diversity in *Phytophthora* species infecting black pepper. 8th International Conference (Hybrid Mode) Plant Pathology: Retrospect and Prospects. organized by Indian Phytopathological Society, (23 -26, March, 2022), SKNAU, Jobner-Jaipur, Rajasthan.

→ Zumaila, F., Jeevalatha, A., & Biju, C. N. 2023. Genetic diversity of *Phytophthora* spp. infecting black pepper as revealed through RAMS and REP PCR analysis. Platinum Jubilee Conference, Plant and Soil Health Management: Issues and Innovations, February 2-4, 2023, Mysuru, Karnataka.

→ Zumaila, F., Jeevalatha, A., & Biju, C. N. 2024. Analysis of genome content and its correlation with growth and fungicide sensitivity of two *Phytophthora* species infecting black pepper. International Seminar on Spices (ISSK) 2024. Spices: Innovative and Green Technologies for Sustainability, June 5-7, 2024, College of Agriculture, Vellayani, Thiruvananthapuram.